INVESTIGATIONS INTO ARSENATE-INDUCED NEURAL TUBE DEFECTS IN A MOUSE MODEL

A Dissertation

by

DENISE SUZANNE HILL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Toxicology

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Approved by:

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ABSTRACT

Investigations into Arsenate-Induced Neural Tube Defects

in a Mouse Model. (December 2007)

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Neural tube defects (NTDs) are malformations affecting about 2.6/1000 births worldwide, and 1/1000 in the United States. Their etiology remains unknown, and is likely due to interaction of genetic susceptibility factors with environmental exposure. Of the many environmental agents considered to potentially contribute to NTD risk, arsenic is one that is surrounded in controversy. We have developed a model system utilizing maternal intraperitoneal (I.P.) exposure on E7.5 and E8.5 to As 9.6 mg/kg (as sodium arsenate) in a normal inbred mouse strain, LM/Bc/Fnn, that is sensitive to arsenate-induced exencephaly. We investigated arsenate induced gene expression changes using DNA microarrays of embryonic anterior neural tube tissue, as well as monitoring of metabolic function in conjunction with the administration of select compounds to rescue the normal phenotype. Finally, to address questions concerning the importance of route of administration and potential maternal toxicity, a teratology study was performed using three arsenate doses administered orally.

Regarding the gene expression study, we identified several candidate genes and ontology groups that may be responsible for arsenate's teratogenicity. Genes include:

engrailed 1 (En-1), *platelet derived growth factor receptor alpha (Pdgfrα)* and *ephrinA7 (EphA7)*. Gene ontology groups identified include oxidative phosphorylation, redox response, and regulation of I-kappaB kinase/NF-kappaB cascade. Acute arsenate exposure induced disruption of mitochondrial function and dependent glucose homeostasis: subsequent hyperglycemia was teratogenic. Maternal treatment with insulin or n-acetyl cysteine, an antioxidant and precursor of glutathione synthesis, proved highly successful in rescuing both the normal phenotype, and to differing degree, the maternal hyperglycemia. Maternal oral arsenate administration also resulted in exencephaly, with exposed embryos exhibiting a positive linear trend with arsenate dosage. There were also linear trends in the relationships between arsenate dose and anomalies involving several components of the axial skeleton: the vertebrae and calvarium. There was no evidence of maternal toxicity as shown by lack of differences in maternal body weight gain, liver, and kidney weights. In conclusion, maternal arsenate exposure (regardless of exposure route) was teratogenic in our model, primarily causing NTDs. Responsible mechanisms may involve disruption of redox and glucose homeostasis as well as expression of established NTD candidate genes.

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have been just as joyful to be a part of yours. I would like to extend a special thanks to Dr. Stanley Glasser, who knows more great stories than anyone since Homer, most of which involve the uterus in some way. His guidance and stories are a welcome daily event.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION

BACKGROUND

Birth defects are a significant health problem worldwide, affecting approximately 6% of births, and resulting in the death of at least 3.3 million children under the age of five each year (1). Major malformations are structure abnormalities that are lethal, require medical or surgical treatment, or are disfiguring. About 2% of newborns are identified as having a major malformation at birth, which occur at similar rates in all species studied, including humans. The causes of structural birth defects are many, and include genetic transmission, chromosomal aberrations, environmental exposures (ionizing radiation), infections, maternal metabolic imbalance, pharmaceuticals and environmental toxicant exposure, with the majority of birth defects having an unknown cause. Types of malformations are investigated individually or clustered based on their shared developmental etiology, cosegregation within families, epidemiologic evidence, or common exposure.

Neural Tube Defects (NTDs)

The second most common type of all structural birth defects, neural tube defects

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This dissertation follows the style of *Diabetes*.

(NTDs), affect approximately 2.6 out of every thousand births worldwide, and 1 per thousand live births in the United States (1; 2). NTDs represent a class of birth defects in which the embryo's neural tube fails to close prior to the end of the first month of pregnancy. Defective closure of the anterior neural tube results in exencephaly or anencephaly, in which the majority of the brain and surrounding tissues are absent. Failure of posterior neural tube closure results in spina bifida, a condition that often results in lower body paralysis and lack of bowel and bladder control (3).

While several distinct causes of NTDs have been identified, the etiology of the vast majority of NTDs remains unexplained. There are numerous environmental exposures that are suspected of inducing NTDs in human embryos, yet definitive conclusions concerning the association between these potentially teratogenic environmental exposures and NTD risk remain difficult to reach. This is, at least in part, due to the relative rarity of both NTDs and documentation of the exposures of interest. A review of the causes of neural tube defects comprises Chapter II.

Folic Acid and Prevention of NTDs

Folates are essential donors in one-carbon metabolism, participating in the biosynthesis of nucleic acids and re-methylation of homocysteine to methionine, a key step for all biomethylation reactions. Many epidemiological and intervention studies have demonstrated that periconceptional folic acid supplementation reduces the occurrence of several congenital malformations, especially NTDs (4; 5). Methyl groups from folates play essential roles in multiple biochemical pathways, and their availability may be

dependent of numerous enzymes in the folate pathway. Thus, there are many potential reactions that could go awry subsequent to disrupted folate metabolism, resulting in aberrant development and the birth of a malformed infant. While folic acid supplementation has proven to have a protective effect for the population as a whole, some subgroups have not benefited to the same degree as the overall population. It is hypothesized that environment, behavior, and genetic variations in folate transport or metabolism may exist, conferring elevated risk and modifying response to periconceptional folate supplementation. This topic is covered more comprehensively in Chapter II.

Evidence for Arsenic-Induced NTDs

Of the many environmental agents suspected of being teratogenic and capable of inducing NTDs, arsenic (As) continues to command intense scientific interest for developmental biologists because studies in both humans and animals indicate that As crosses, and may accumulate in, the placenta at concentrations exceeding that of the maternal blood (6; 7). While it is a known laboratory animal teratogen (8-16), the few epidemiological studies of reproductive outcome following maternal As exposure are insufficient for properly assessing its teratogenic potential in humans (17-25). Due to the relative rarity of specific malformations, available studies have employed insufficient sample sizes to demonstrate associations with specific birth defects (instead, generally describing observed malformations being as major or minor). In addition, the majority of human epidemiological studies have relied on proxy measures of exposure (e.g. distance

from smelter) that are subject to varying degrees of misclassification (26). In the few studies in which maternal As exposure was directly evaluated, measurements were not taken during the relevant embryological period of neural tube closure.

The best evidence currently available falls short of being able to conclude definitively that As is not a human teratogen: it remains an important and, as yet, unresolved issue. On the contrary, despite inconclusive results, evidence suggests that there may well be a relationship between human *in utero* As exposure and adverse pregnancy outcomes. Acute high dose and chronic low dose As exposure during pregnancy has been associated with increased pre- and post-natal mortality (13; 27; 28). In addition, chronic low dose As exposure has been associated with low birth weight and developmental impairment (13). While small study sample size makes it difficult to demonstrate associations with specific malformations, several studies have suggested an association between maternal exposure to As and general malformations in the offspring.

At least two studies have proposed an association between maternal As exposure in drinking water and increased risk of congenital heart malformations in exposed offspring (22; 29) The first study explored the relationship between the population-weighted mean arsenic concentration in public drinking water supplies, and mortality from circulatory diseases in 30 U.S. counties from 1968 to 1984. Mean arsenic levels ranged from 5.4 to 91.5 micrograms/l. Standard mortality ratios (SMRs) for congenital anomalies of the heart tended to be elevated for counties exceeding 20 micrograms/l. In the second study, information concerning 270 cases and 665 control children was used to identify maternal exposures to drinking water contaminants associated with increased

risks for congenital heart disease. Arsenic exposure at any detectable level was associated with a threefold increase in the occurrence of coarctation of the aorta (prevalence odds ratio = 3.4, 95% confidence interval = 1.3-8.9). Finally, a recent case-control study of the association between maternal exposure to heavy metals and the risk of NTDs identified elevated odds ratios for the association between NTDs and each measure of maternal As exposure that was assessed (30). These include maternal occupational exposure to arsenic, OR=1.5, 95% CI 0.5-8.8, drinking water arsenic >10 μg/l OR=2.0, 95% CI 0.1-3.1, and maternal address at conception within two miles of a facility with arsenic emission OR=1.2, 95% CI 1.2 (0.3-4.3). In addition, urinary arsenic levels greater than or equal to 38.3 μg/l, which was at or above the 95th percentile of controls, were detected in 4/70 case women and none of 56 control women. While none of the associations in this study achieved statistical significance, a potential association between maternal As exposure and NTD risk cannot be excluded due to the relatively small study sample (n=225 case women), and the consistency of the results across several different measures of As exposure. Indirect evidence for an association between As and NTDs is provided by studies of maternal pesticide exposure. While arsenic exposure is often associated with industry, arsenic is also commonly used in pesticides, and epidemiological studies suggest that NTD risk is associated with maternal exposure to pesticides or proximity to agricultural areas (13; 31; 32). Given that As is environmentally persistent, and continues to be used in a range of agricultural products (e.g. pesticides, especially herbicides, growth enhancers, etc.), these studies provide a potential link between As and NTD risk.

Additional evidence that maternal As exposure may be associated with an increased risk of congenital malformations, primarily NTDs, is provided by studies conducted using animal models. These studies have demonstrated that As preferentially accumulates in the neuroepithelium of the developing embryos of hamsters, mice and monkeys (33; 34), and that maternal As exposure is teratogenic. Furthermore, As is associated with an increased risk of NTDs when the exposure occurs specifically during the period of neural tube closure.

Arsenic Metabolism and Toxicity

There are millions of people worldwide exposed to arsenic, at levels thought to have adverse health effects, primarily from contaminated water sources (Table 1.1). A well-compiled description of exposure levels that exist in various countries was provided by Nordstrom (35). Epidemiology studies have generally been inconsistent in the arsenicals measured. Some studies have measured total arsenic exposure; these studies often use the general nomenclature ‘arsenic’, or ‘As’ (29; 36). Others have focused on inorganic arsenicals. Inorganic trivalent (AsIII) and pentavalent (AsV) arsenicals were thought to be the only biologically relevant arsenicals, and these studies have generally used the nomenclature ‘inorganic arsenic’ or ‘Asi’, infrequently distinguishing relative levels AsV and AsIII (37; 38). For this reason, much of the commentary in this dissertation, and in arsenic research as a field, concerning the large body of early investigation into

Table 1.1 Summary of human health effects from arsenic exposure. Adapted from Carter et al (39).

Health effect	Dose (μg/kg/day)
Blood hemoglobin increases	1-10
Hypertension	4
Prostate cancer risk	4
Circulatory changes	4
Urinary porphyrin increases	9-17
Melanosis	6-20
Hyperkeratosis	6-20
Peripheral vascular alterations	12
Gangrene of the extremities	25-35
Hematology effects	40
Distal polyneuropathy	40
Anemia	50
Prominent peripheral neuropathy	300
Diabetes	?

arsenic exposure and potential increased risk of certain adverse human health outcomes, is limited to descriptions of risk associated with arsenic exposure, as either As or Asi.

In the context of the elucidation of the various arsenical's toxicity, this is understandable. Dramatic advances in the field of arsenic research have taken place over the past decade. Only recently has evidence been revealed to indicate that biotransformation involving methylation of inorganic arsenic species to organic species did not confer detoxification. Some of the most significant advances have been established by landmark papers in the field, including: classic work on biotransformation of inorganic arsenic species both in vitro and in vivo (40; 41), biotransformation of inorganic arsenic (42), the relative toxicities of trivalent monomethylated species (MMA) as compared to arsenite (43), the revelation that methylation is not a

detoxification mechanism (42), proposed ranking of arsenicals by relative toxicity (44), the discovery of MMAIII and DMAIII in human urine (45).

It is well established that oral exposure to either arsenite or arsenate results in excretion of all forms arsenic, both inorganic and methylated arsenicals (40; 41). While the exact mechanisms involved in the series of reduction and subsequent methylation reactions are still being elucidated, a number of promising candidates have been revealed: reduction by glutathione (46) or a number of proposed dithiols, in addition to superoxide and H_2O_2 (47), and oxidative methylation by an arsenic methyltransferase, Cyt19, a SAM-dependent methyltransferase suggested to work on arsenic-GSH complexes (48). A recent hypothesis has proposed the reduction of arsenicals by superoxide and H_2O_2, and suggested to replace the current hypothesis wherein the reduction of arsenate to arsenite is achieved by a nonenzymatic reaction with GSH.

The classical primary mammalian metabolic sequence for AsV is as follows: reduction to AsIII, biomethylation to monomethylarsonic acid (MMAV), reduction to MMAIII, and biomethylation to dimethylarsinic acid (DMAV), which is often the major arsenic metabolite excreted in the urine (40). Reduction reactions are thought to require glutathione, whereas the biomethylation reactions are catalyzed by SAM-dependent methyltransferases (49; 50). SAM is eventually regenerated by remethylation through the homocysteine-methionine cycle, a process that requires 5-methyl-tetrahydrofolate (M-THF) as a co-factor (Figure 1.1).

In the conventional metabolic pathway of arsenate metabolism, pentavalent metabolites are reduced to the more toxic trivalent compounds. Hayakawa et al.(Figure

1.2) has proposed a pathway in which the +3 arsenic species are formed (by oxidation) before the +5 species, which are the ultimate products of arsenic metabolism (48). This new pathway is in agreement with Aposhian's observation that ''oxidation is detoxification of arsenic'' (47).

In a comparison of the relative toxicity of arsenate and arsenite to various rat and human cells, relative toxicity of arsenicals was summarized as follows: trivalent methylated arsenicals were significantly more toxic than pentavalent methylated arsenicals. MMAIII was more toxic than arsenite, and DMAIII was at least as toxic as arsenite for most cell types examined (44).

In a study by Hughes et al. comparing the relative toxicities of MMAIII and MMAV administered orally in a mouse model, it was found that the profile of arsenicals excreted in the urine and feces was different between the two treated groups. In brief, methylation of MMAV was limited, while methylation of MMAIII was extensive, with less than 10% of the dose excreted in urine following MMAV treatment was in the form of methylated products, while is was greater than 90% following MMAIII treatment. For the interested reader, a comprehensive overview of results from similar studies in various animal models is provided by Hughes (51).

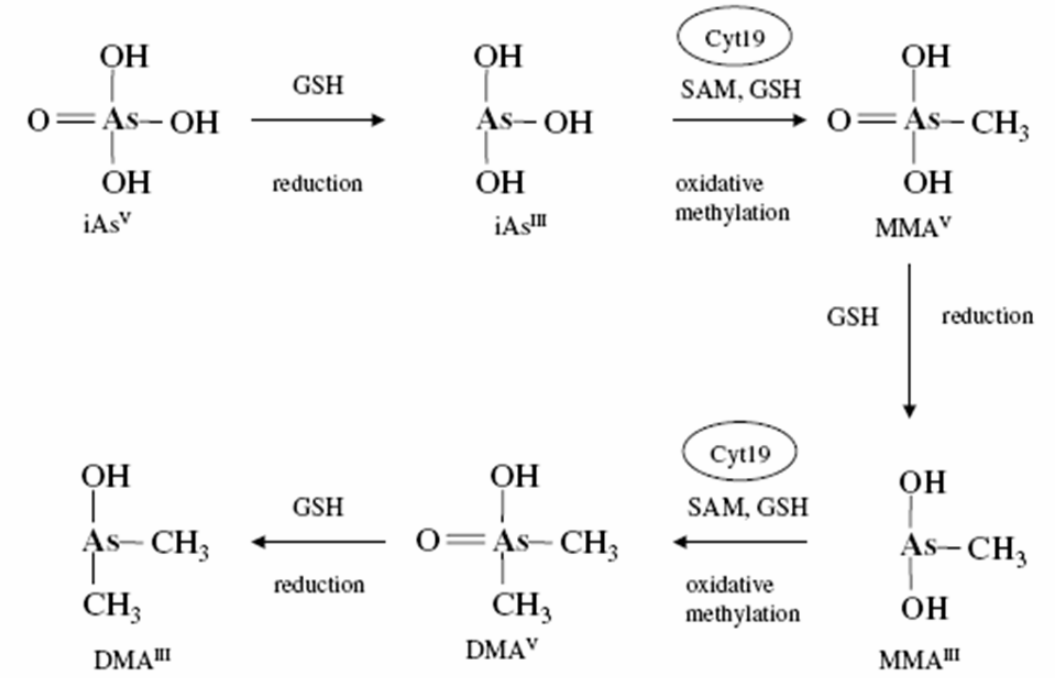

Figure 1.1. The classical metabolic pathway of inorganic arsenate. From Hayakawa et al (48). iAsIII arsenite, iAsV arsenate, MMAIII monomethylarsonous acid, MMAV monomethylarsonic acid, DMAIII dimethylarsinous acid, DMAV dimethylarsinic acid, Cyt19 arsenic methyltransferase, GSH reduced glutathione, SAM S-adenosyl-L-methionine)

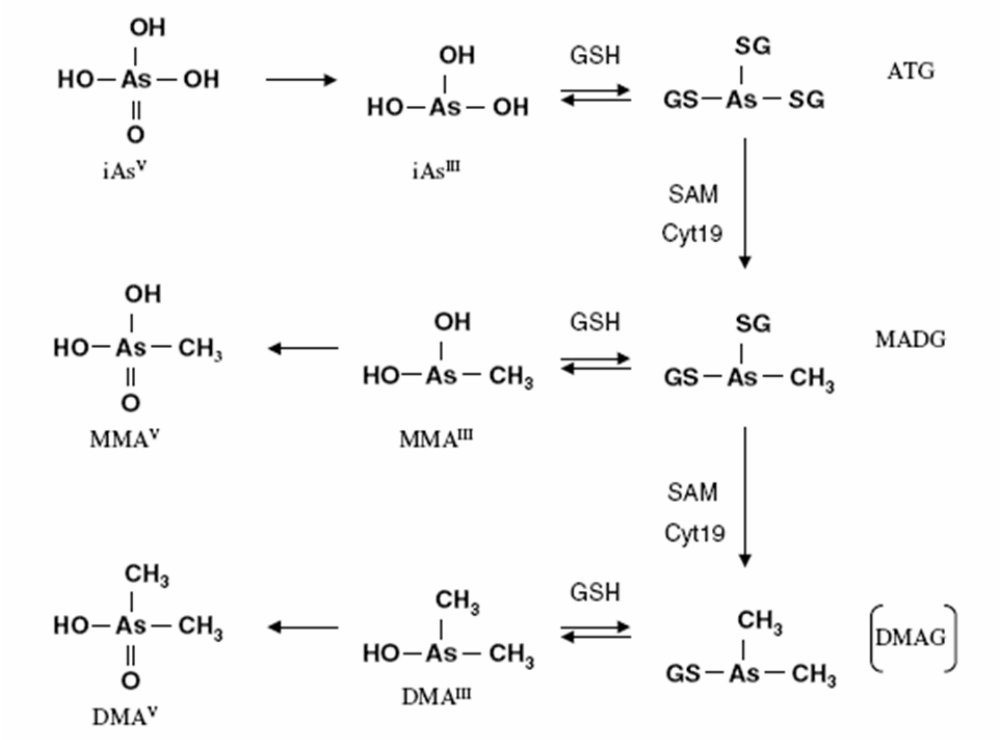

Figure 1.2. A proposed metabolic pathway of inorganic arsenate. From Hayakawa (48). iAsIII arsenite, iAsV arsenate, GSH reduced glutathione, AGT arsenic triglutathione, SAM Sadenosyl- L-methionine, Cyt19 arsenic methyltransferase, MADG monomethylarsonic diglutathione, MMAIII monomethylarsonous acid, MMAV monomethylarsonic acid, DMAG dimethylarsinic glutathione, DMAIII dimethylarsinous acid, DMAV dimethylarsinic acid.

While a characterization of the relative toxicities of arsenic and elucidation of the various mechanisms of toxicity is still evolving (Table 1.2), considerable attention has been paid to this topic during the past decade, and a comprehensive and thoughtful review has been published by Carter et al. (39). The work is briefly summarized here. Generally, as mentioned, trivalent arsenic species are more toxic than the pentavalent species. Individual arsenicals need greater characterization in terms of their toxicity and mechanisms: there are six different urinary metabolites, and three different GSH-AsIII complexes, as well as an As-selenium compound. Methylation is not considered to be a detoxification pathway, nor does it increase solubility (though it may prevent methylated compounds from entering cells). The trivalent compounds arsine (arsenic trioxide) and gallium arsenide have important industrial applications. Currently, the best characterized arsenicals, in terms of toxicity, are arsenite and arsenate. The suggested mechanisms conferring arsenite's toxicity include inhibition of pyruvate dehydrogenase (PDH), binding to thiols, and transfer of electrons in oxidation-reduction reactions. It has also been suggested to be genotoxic, and cause disruption of the cellular cytoskeletal elements. Arsenate may be reduced to arsenite, and it has been suggested that arsenite may be the arsenical responsible for the cytotoxic effects following arsenate treatment (52). Arsenate is suggested to possesses a distinct toxicity due to its similarity to phosphate: arsenate is identical to phosphate in structure and acid dissociation constants,

Table 1.2. Arsenic species important in toxicity. Adapted from Carter et al. (39).

Name (formula)	Chemical form	Comment
$As(OH)_3$, arsenious acid, often called arsenite	Oxidation State, AsIII	Aqueous solution species for +III. pKa= 9.3,12.1, 13.4
H_3AsO_4, arsenic acid usually called arsenate	Oxidation state, AsV	Aqueous solution species for +V at pH 7.4 is -2 charged anion ($HAsO_4^{-2}$) pKa= 2.2, 6.98, 11.5
AsH_3, arsine	Oxidation state, As(-II) Hydride (H^-)	Most reduced form of arsenic, slightly soluble in water.
CH_3-$As(OH)_2$, monomethylarsonoic acid (MMAV)	Oxidation state is +V	Methylated metabolite of AsIII, metabolized to dimethylarsinic acid
CH_3-$As(OH)_2$, monomethylarsonous acid (MMAIII)	Oxidation state is +III	Reduced metabolite of MMAV
$(CH_3)_2$-As(O)(OH), dimethylarsinic acid, (DMAV)	Oxidation state is +V	Dimethylated metabolite of AsIII and methylated metabolite of MMAIII, pKa=6.2
$(CH_3)_2$-As(OH), dimethylarsinious acid (DMAIII)	Oxidation state is +III	Reduced metabolite of MMAV
GaAs, gallium arsenide	Oxidation state of As is 0 to -III	Synthetic compound with no acid-base behavior

and thus exclusively substitutes for phosphate in reactions. The implications of this are described more comprehensively in the section concerning the mitochondriotoxicity of inorganic arsenicals.

Mitochondrial Development and Its Relationship to NTDs

During neural tube closure, the concurrent onset of embryonic oxidative phosphorylation and cardiac function is a highly coordinated event involving maturation of mitochondrial cristae and a related increased dependence on oxygen supply. It is clear that glucose plays a significant nutritional role in early embryonic development. The high glucose

utilization of anaerobic glycolysis, essential to the early embryo, drops as the activity of the Kreb's cycle and electron transport increases. (53) While mitochondrial morphology and function during embryogenesis is not well characterized, there is an early developmental pattern characterized by vesiculation of the mitochondrial cristae, in which the components of the electrons transport chain are embedded. During early organogenesis, the embryo is in a relative state of hypoxia, which is associated with decrease of electron transport system activity (and oxidative phosphorylation) and a marked increase in glycolysis. Shepard suggests that the delamellated state of the cristae is associated with quiescence of the electron transport chain, and is present in early embryogenesis in order to protect the embryo from toxic respiratory end-products of oxidative respiration which could accumulate in an embryo lacking vascular perfusion (54).

There is a clear pattern indicating that disruption of mitochondria during neural tube closure may result in NTDs. Treatment with antimycin A, which inhibits complex 3, on E7.5 causes NTDs in mouse embryos (55). Acrylonitrile is demonstrated to be converted in vivo to toxicologically significant concentrations of cyanide, which inhibits complex 4. Acrylonitrile administered to hamsters by I.P. injection at 80-120 mg/kg on E8 results in exencephaly of the offspring (56). UCP2, which uncouples oxidative phosphorylation from the production of ATP by permitting hydrogen ions to bypass ATP synthase, is a known regulator of blood glucose. A known risk for NTDs, gain-of-function deletion mutations or SNPs in UCP2, increases the risk of spina bifida (OR>3.6) (57). Engineered mouse knockouts for Thioredoxin 2 (Txn2), a mitochondrial

antioxidant, are embrolethal, with failure of anterior neural tube closure. Similarly, knockouts for Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), which are essential for normal mitochondrial dynamics, are both embryolethal (58). However, while Mfn1 fetuses display normal morphology, Mfn 2 knockouts fail to close their anterior neural tube (59).

Arsenic-induced Mitochondrial Disruption and Its Importance to Redox Homeostasis and Neural Tube Closure

Although the toxicity of arsenic varies based on its chemical form and oxidation state, mitochondria are a consistent and primary target in both environmental exposure and laboratory models. Cellular disruption caused by mitochondrial reactive oxygen species (ROS) are thought to underlie much of arsenic's inherent toxicity. Krebs first described arsenite's disruption of pyruvate metabolism in the 1930's (60), and it has since been demonstrated to inactivate nearly all of the Krebs cycle enzymes, resulting in mitochondrial release of H_2O_2 (61-65). Much of the work in this field has been driven by the rediscovery of arsenic as a pharmaceutical, with its current primary medical use as a chemotherapy for recurrent or ATRA-resistant leukemia, in the form of Trisenox (arsenic trioxide) (66-68). While trisenox is intended for use in humans, much published research concerning its hypothesized mechanism of toxicity is performed in vitro, which as previously mentioned, may confer differences in toxicity and mechanisms due to differences in pharmacokinetics in comparison to in vivo studies.

It is unknown as to what degree arsenate's toxicity is due to biotranformation to the more toxic arsenite. Unlike arsenite, which freely crosses the cell membrane, arsenate is a charged salt, and has to be transported into the cell. As a phosphate analog, it concentrates in the mitochondria (69; 70). It has even been suggested that mitochondria reduce arsenate to arsenite: they have been observed, in culture, to take up arsenate, rapidly reduce it, and export all of the arsenate as arsenite into the media (71). The hypothesized reduction of arsenite to arsenate may play a part in this observed phenomenon. Its preferential accumulation in mitochondria, and its biotransformation to the known mitochondriotoxin, arsenite, may account for some of its observed mitochondriotoxicity. Arsenate may exert profound mitochondriotoxicity through substitution for phosphate in glycolytic and cellular respiration pathways, although it is unknown whether arsenolysis of ATP occurs in vivo (70). Like arsenite, arsenate has also been found to decrease pyruvate dehydrogenase (72). A novel finding described in detail in Chapter III, an arsenate salt, sodium arsenate, has been demonstrated to decrease expression of genes encoding structures contributing to complexes of the electron transport chain (61). If ATP production is reduced significantly, it causes profound mitochondrial disruption and subsequent release of H2O2 into the cytosol and cellular disruption. Interestingly, much of the work concerning arsenate's mitochondriotoxicity was discovered via its use as a phosphate analog to compete with cellular phosphate in order to deplete ATP production (73-78).

Arsenic metabolism and excretion is primarily dependent on the depletable substrate, GSH. Additionally, GSH is utilized in the dismutation of H_2O_2 to molecular

oxygen and water. Thus, arsenic exposure both causes significant H_2O_2 stress, while simultaneously impairing the antioxidant system essential for its neutralization. The metabolism and excretion of heavy metals such as arsenic depends on the presence of antioxidants and thiols that aid arsenic methylation, and arsenic metallothionein-binding. Induction of oxidative stress occurs through many of these pathways, such as the oxidative stress-induction of metallothionein and superoxide dismutase that produce their own free-radical waste, inducing further oxidative damage.

It is well established that partially reduced oxygen species shed from mitochondria have a variety of diverse consequences for the cell. In its simplest description, when the production of these reactive oxygen species (ROS) outstrips cellular antioxidant defenses, oxidative stress results (79-81). ROS disrupt cells through two main pathways: physical damage, and altered cellular signaling. In addition, damage from ROS may initiate a feedback loop, causing continued cellular damage. Single exposures to some oxidative stress-inducing xenobiotics may result in prolonged oxidative stress responses (82). All classes of cellular macromolecules face ROS induced damage. Lipid peroxidation may result in altered membrane fluidity, permeability, and transport characteristics. Hydroxylated bases, DNA single strand breaks (DNA-SSBs), and chromosome breaks may all lead to faulty transcription, resulting in cell injury, and possibly cell death (83).

Cell signaling that may be altered though changes in redox state, alteration in cellular membrane transport, oxidation states of metallic cofactors, and ion homeostasis (84; 85). Altered cellular redox states are shown to disrupt the expression of redox

sensitive transcription factors NF-kB and HIF-1α (85), and free radical adducts have been shown to induce methylation of adjacent cytosines, a response that displayed a high degree of positional specificity and may account for some fraction of the gene specific methylation observed that likely to be the more important aspect of general, global, hypomethylation (86).

Emerging evidence suggests that oxidative signaling and redox-sensitive signal transduction pathways are critical for regulating basic developmental processes such as proliferation, differentiation, and apoptosis (87). Therefore, chemicals that disrupt redox homeostasis may induce teratogenesis via the misregulation of these same pathways. Thiol redox couples are key regulators of redox homeostasis, and include glutathione/glutathione disulfide, thioredoxinred/thioredoinox, and cysteine/cystine. (87). Redox disruption may occur as the result of developmental pathologies or toxicant exposure, which is often associated with ROS or RNS formation (88).

Diabetes Classification and Diagnosis

Diabetes' cause and treatment is of intense interest, and the field has advanced accordingly. Relevant portions of the American Diabetes Association's 2007 position statement on diagnosis and classification of diabetes mellitus (89) is briefly summarized here. Diabetes mellitus is a heterogeneous group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or some combination of the two. Pathogenic processes that are observed to be involved in the development of diabetes include autoimmune destruction of pancreatic ß-cells and

subsequent insulin deficiency, as well as abnormalities that result in resistance to insulin's action. Resistance to insulin's action may result from diminished tissue response to insulin, as well as, paradoxically, inadequate insulin secretion. Both may occur in the same patient, and definitive establishment of the primary cause of hyperglycemia is often unclear. The vast majority of cases of diabetes fall into two broad categories: type 1 and type 2 diabetes. Type 1 diabetes is caused by deficiency of insulin secretion, which accounts for about 5-10% of cases. It has previously been described as insulin-dependent diabetes, type I diabetes, or juvenile-onset diabetes. The immune-mediated diabetes results from autoimmune destruction of the pancreatic ß-cells, and may be identified by markers of immune destruction, which are present in 85-90% of individuals when fasting hyperglycemia is initially detected. Although autoimmune destruction of ß-cells has been associated with variants within multiple genes, and is related to some identified environmental factors, reasons for onset are still poorly understood.

Type 2 diabetes accounts for the vast majority of cases (90-95%) and has previously been described as non-insulin dependent diabetes, type II diabetes, and adult-onset diabetes. This type of diabetes is characterized by insulin resistance, and patients also usually have relative insulin deficiency. Risk increases with age, obesity, lack of physical activity, prior gestational diabetes, established hypertension or dyslipidemia, and occurs with varying frequency in different racial and ethnic subgroups. While it is associated with a strong genetic predisposition, the etiology is complex and not well defined.

Other specific types of diabetes include: genetic defects of the ß-cell, genetic defects of insulin action, diseases of the exocrine pancreas, endocrinopathies, drug- or chemical-induced diabetes, infections, gestational diabetes, and other genetic syndromes sometimes associated with diabetes.

Three ways to diagnose diabetes are possible, and in the absence of unequivocal hyperglycemia, the diagnosis must be confirmed on a subsequent day by any of the three methods. These methods include: a fasting plasma glucose (FPG) level (no caloric intake for at least 8 hours) greater than 126 mg/dl (7.0 mmol/l), a casual plasma glucose level greater than 200 mg/dl (11.1 mmol/l), or a 2-hour postload glucose greater than 200 mg/dl (7.0 mmol/l) following an oral glucose tolerance test (OGTT) using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water.

Induction of Diabetes by Pharmaceuticals and Environmental Contaminants

Many pharmaceuticals, household, and industrial chemicals can impair insulin secretion. While these exposures do not cause frank diabetes, they may initiate diabetes onset in individuals who are predisposed, such as those with insulin resistance. It is difficult to classify such cases as to type of diabetes. While both rare and extreme, exposures to Vacor or intravenous pentamidine can permanently destroy pancreatic β-cells, causing an insulin-dependent diabetes that mimics type 1 diabetes. Additionally, α-interferon treatment has been reported to result in diabetes with antibodies to islet cells. Other pharmaceuticals may impair insulin action, such as nicotinic acid and glucocorticoids.

A sampling of pharmaceuticals and compounds recognized to induce diabetes (and application) include: Vacor (rat poison), pentamidine (antimicrobial), nicotinic acid (vitamin b_3), glucocorticoids (steroid), thyroid hormone, diazoxide (vasodilator), β-adrenergic agonists (calcium channel modifiers), thiazides (diuretic), valproate (antiepileptic), α-interferon (cancer and viral therapeutic). Interestingly, thiazides are associated with NTDs, and dilantin is associated with cleft palate, another craniofacial malformation associated with diabetic pregnancy (89). Valproate, a known risk factor for NTDs, is an antiepileptic and atypical antipsychotic, which are widely observed cause hyperglycemia (90; 91).

The first environmental contaminant to be associated with an increased risk of diabetes was found in the 1970s, when carbon disulfide exposure's relationship to diabetes was reported (89; 92). Interest in other environmental contaminants and their potential to increase diabetes risk is relatively recent, and has focused on arsenic, dioxin, and nitrates (93-96). For a comprehensive review of this subject, see work by Longnecker (97). At the time of publication of Longnecker's work, while no exposures were conclusively linked with increased diabetes risk, several occupations and occupational exposures were identified that were thought to have contributed to diabetes onset. Evaluation of studies was hampered aspects of study design that limit the power of the investigation including: use of glucosuria or diabetes death as diagnostic criteria, lack of adjustment for possible confounders in some studies, and failure to consider both type 1 and type 2 diabetes as possible adverse health outcomes. Data concerning Arsenic and TCDD were most suggestive of an association with diabetes, while nitrates, nitrites,

and N-nitroso compounds had a weaker, but not null, association. Further epidemiological and laboratory research concerning arsenic's association with increased diabetes risk has since been published (98-105).

Arsenic Exposure and Glucose Homeostasis

Multiple epidemiological studies show that arsenic exposure is a risk factor for type 2 diabetes in some populations, both in areas where people are exposed through contaminated drinking water, as well as in areas that are relatively free of environmental pollution and where exposures are primarily occupational (94; 95; 106-108). Deliberate exposure to various arsenicals causes hyperglycemia in humans and rodents. Paradoxically, exposure may also cause hypoglycemia. The reasons for this are an area of intense research, as investigators struggle to understand the complexities of glucose homeostasis. The most recent research suggests that pancreatic beta cells have a bimodal response to oxidative signaling, wherein very low levels of H2O2 from normal glucose driven mitochondrial function results in insulin release and a subsequent lowering of blood sugar, but severe oxidative stress/damage may decrease □-cell function and insulin release, resulting in hyperglycemia (109).

In humans, one of the most common side effects of arsenic trioxide treatment as a cancer chemotherapeutic is hyperglycemia (66). Similarly, acute and sub-chronic exposure to arsenite is observed to elevate blood sugar concentrations in rodents and goats (103; 110-113). The studies suggest the development of diabetes mellitus after long term, sub-lethal arsenic exposure with a disease pathology that recapitulates type 2

diabetes. Arsenite exposure has also been observed to cause hypoglycemia in some a rat model (114; 115) Arsenate has been the subject of few studies, but a similar pattern has been observed. Arsenate exposure has been observed to cause hyperglycemia in a □-cell model (116) as well as hypoglycemia (117) in a rodent model.

The mitochondrial release of H2O2, thought to underlie much of arsenic's toxicity provides a mechanistic link to disrupted glucose metabolism. Mitochondrial oxidative phosphorylation (OxPhos) links circulating glucose and insulin levels via ATP-dependent glucose stimulated insulin secretion (GSIS). Arsenic has been demonstrated to inhibit GSIS (Figure 1.3) *in vitro* when administered to isolated pancreatic β-cell islets under glucose challenge (104). Additionally, excess hydrogen peroxide has been observed to reversibly repress many Krebs cycle enzymes, making pancreatic β-cells insensitive to GSIS, as demonstrated in *vitro*, as well as *in vivo* models (118-120).

Agents that are toxic to mitochondria are regularly used in the clinical treatment of cancer and AIDS, commonly resulting in disruption of energy metabolism and hyperglycemia (121; 122). This may be due to failure of GSIS, as ATP is necessary for several steps in this pathway, such as phosphorylation of the glucose molecule,

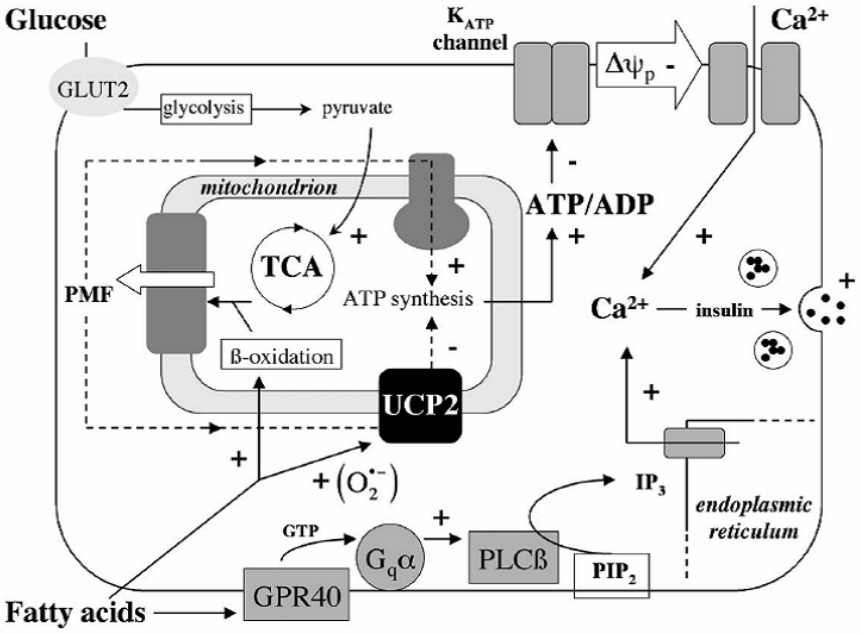

Figure 1.3. Glucose stimulated insulin secretion. From Brand et al. (123). Oxidative phosphorylation links glucose stimulus and insulin secretion. Glucose equilibrates across the plasma membrane via the glucose transporter (GLUT2), driving glycolysis. Glucose oxidation establishes the proton motive force (PMF) that powers the transport of protons out of the matrix into the intermembrane space to create a proton gradient. The inner membrane is impermeable to protons, and only a few structures exist that will allow the protons to pass, such as ATP synthase, and UCP2. The proton gradient is harnessed by ATP synthase, producing ATP, and increasing in the ATP:ADP ratio. This causes closure of K_{ATP} channels, depolarization of the plasma membrane potential (Dcp), and allowing opening of the voltage-sensitive Ca_2+ channels, and calcium influx. This increase in cytosolic Ca_2+ is thought to be the main trigger for exocytosis of insulin. At permissive glucose concentrations, fatty acids may potentiate GSIS through a G-protein-linked receptor (GPR40) pathway.

potassium channel binding, and exocytosis of insulin granules. One of the best studied of these compounds is arsenic trioxide, a cancer therapeutic. Arsenic trioxide induced cell death involves an early decrease in the normal cellular mitochondrial membrane potential and a subsequent increase in cytosolic ROS content, predominantly H_2O_2. Efficacy is augmented by co-treatment with compounds that increase hydrogen peroxide stress, such as those that inhibit glutathione, the key substrate dismutating H_2O_2.

A byproduct of mitochondrial function, hydrogen peroxide is a negative feedback inhibitor of the Krebs cycle, preventing a toxic rise in oxidative radicals by controlling the rate of mitochondrial oxidative metabolism (118; 119; 124). H_2O_2 has a signaling utility that is especially potent in B-cells: they possess a weak antioxidant enzyme defense system, especially with regard to hydrogen peroxide-decomposing enzymes (125; 126).

Hyperglycemia bypasses the metabolic use of the pentose shunt (Figure 1.4), which is necessary for production of glutathione, and thought to underlie diabetes' observed oxidative stress component. Therefore, as glutathione is necessary for arsenic metabolism and excretion, hyperglycemia-induced inhibition of glutathione production may potentiate arsenic's toxicity. This creates an interaction wherein mitochondrial damage results in the release of oxidative radicals, which subsequently results in hyperglycemia, thereby limiting the antioxidant capacity to protect the organism from further mitochondrial damage.

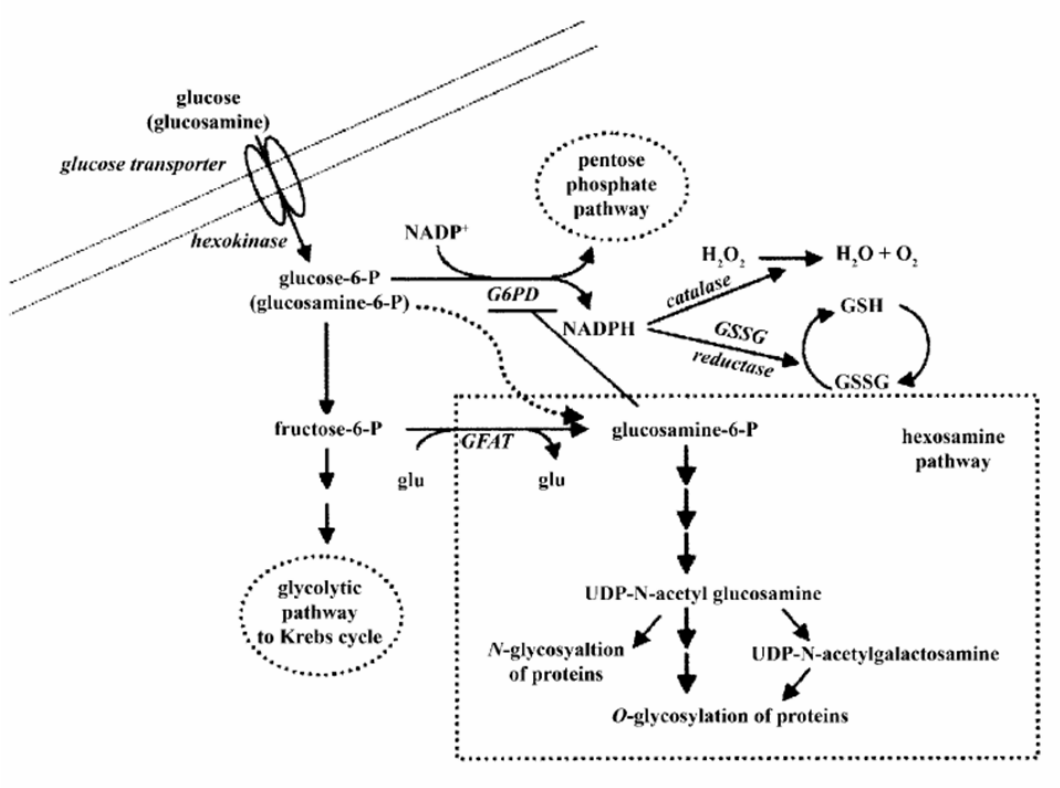

Figure 1.4. Activation of the hexosamine synthetic pathway by increased glycolytic flux. From Horal et al. (127). The rate limiting enzyme for hexosamine synthesis is glutamine:fructose-6-phosphate amidotransferase (GFAT), which yields glucosamine-6-phosphate: select downstream metabolites are shown. UDP-N-acetyl glucosamine and UDP-N-acetyl galactosamine are substrates for O-glycosylation of proteins. Glucosamine-6-phosphate inhibits activity of glucose-6-phosphate dehydrogenase (G6PD), the rate limiting enzyme of the pentose shunt pathway. G6PD activity is coupled to production of NADPH, which, in turn, is coupled to glutathione reductase (GSSG reductase) and enhances catalase activity. Thus, hyperglycemia-induced increased flux through the hexosamine pathway ultimately results in a reduction of available antioxidant substrates catalase, and more importantly, glutathione. The hexosamine pathway can be directly activated experimentally by administration of glucosamine.

Maternal Periconceptional Hyperglycemia and NTDs

Maternal periconceptional hyperglycemia is teratogenic (128-130). Maternal blood glucose freely crosses the placenta. The developing human embryo lacks pancreatic function until well after neurulation is complete, and thus equilibrates with the maternal circulating glucose. As half of all pregnancies are unplanned, and women who have missed their period have already passed the critical time of neural tube closure, good glycemic control in pre-existing diabetic pregnancies reduces the risk of NTDs (131-133). In rodents, maternal hyperglycemia induced by direct injection of glucose or B-cell disruption from streptozotocin treatment results in exencephaly in the offspring, and embryonic explants grown in high glucose media also exhibit failure of neural tube closure (134). A growing body of research conducted over the past decade suggests that maternal hyperglycemia induces teratogenic oxidative stress in the exposed embryo, which is consistent with the well-established relationship between diabetes and oxidative stress (135).

As and Perturbation of Methylation

The mechanistic relationship between arsenic exposure and disruption of many aspects of one-carbon metabolism is the subject of much investigation. The areas of most intense interest include disruption of the SAM/SAH remethylation cycle, and disruption of epigenetic methylation in the promoter regions of genes.

It is plausible to suggest that diversion of methyl groups to arsenic's biotransformation and excretion interferes with the SAM/SAH remethylation cycle,

resulting in accumulation of homocysteine. It is well established that elevated homocysteine is associated with impaired biotransformation and excretion of arsenic, exposing the affected individuals to arsenicals at increased levels (136). Additionally, homocysteine is known to cause NTDs in chick embryos (137). Furthermore, it is suggested that homocysteine and H_2O_2 act synergistically to increase mitochondrial damage, already identified as one of the main cellular organelles targeted by arsenic toxicity (138; 139).

Arsenic has been demonstrated to induce both DNA hypomethylation and chromosome instability, as well as gene-specific hypermethylation (140-146). The direct causes for this are still being investigated by multiple investigators, though some mechanisms have recently been proposed. As previously described, arsenate is metabolized via biomethylation to MMAV and DMAV. Elevated *in vivo* AsV concentration requires considerable amounts of SAM for its biotransformation and excretion, and is likely to temporarily deplete SAM pools. Altered DNA methylation could result in changes in gene expression that could have dire consequences for the exposed embryo. In addition, even temporary depletion of SAM may adversely affect arsenic's major route of metabolism and removal from the body, biomethylation, elevating and prolonging concentration of the more toxic trivalent arsenicals, As III, MMAIII, and DMAIII, *in vivo*.

CHAPTER II

INVESTIGATIONS INTO THE ETIOLOGY OF NEURAL TUBE DEFECTS*

OVERVIEW

Neural tube defects (NTDs) are serious malformations affecting approximately 1 per 1000 births, yet the mechanisms by which they arise are unknown. There have been consistent efforts in many fields of research to elucidate the etiology of this multifactorial condition. While no single gene has been identified as an independent risk factor for NTDs, candidate genes have been proposed that may modify the effects of maternal and/or embryonic exposures. Folate supplementation effectively reduces the occurrence of NTDs and, consequently, has focused much research on metabolism of folate-related pathways during pregnancy and development. Further understanding of normal development and how teratogens can perturb these orchestrated processes also remains at the fore of modern scientific endeavors. The composite of these factors remains fragmented; the aim of this review is to provide the reader with a summary of sentinel and current works in the body of literature addressing NTD disease etiology.

INTRODUCTION

Like any serious congenital malformation, neural tube defects (NTDs) have adverse consequences that afflict society as well as the affected individuals. The emotional

* Reprinted with permission from " Investigations into the etiology of neural tube defects" by Cabrera R.M., Hill D.S., Etheredge A.J., Finnell R.H., 2007. *Birth Defects Res C Embryo Today*, 72:330-44, Copyright (2004) by Wiley.

burden and monetary expenditures associated with these congenital malformations has focused research towards understanding those underlying mechanisms of normal development and how teratogens can perturb these highly regulated processes. In this regard, the term teratogen implies that the agent alone alters development, while in reality; teratogenicity is the outcome of multiple factors. These factors not only involve the physical and chemical nature of the agent, but also the dose, route and gestational timing (147). NTDs are known to occur in one out of every thousand pregnancies in the United States (148), with varying rates reported among the world's populations (149-151). With an aim towards expanding our understanding of the mechanisms underlying the etiology of these devastating defects, numerous studies have been carried out on the etiology of both teratogen-induced and spontaneously occurring NTDs.

Neurulation and Neural Tube Defects

The embryonic period, week three through eight in human development, is the time frame during which organogenesis occurs (152). It is also the most susceptible period for induction of malformations by teratogens (153). The most common and severe malformations of the central nervous system that can occur during this time are NTDs. These malformations include those anomalies that involve failure of the neural tube to close during the 4^{th} week of embryogenesis, and the most prevalent NTDs are anencephaly and spina bifida. Neurulation occurs during the gestational time period between the formation of the neural plate and the closure of the neural tube, the latter being the precursor of both the central and most of the peripheral nervous system.

Neurulation can also be divided into primary and secondary processes. During primary neurulation, fusion of the neural folds involves shaping and folding neural ectoderm and the interposition of mesenchyme. As midline fusion of the neural and surface ectoderm proceeds, the neural tube is formed and covered by surface ectoderm; accordingly, the neural ectoderm becomes located interiorly. Secondary neurulation creates the lowest portion of the spinal cord, from the upper sacral to the coccygeal regions. At the caudal levels of neural tube development, a tail bud or caudal eminence is formed. The tail bud is the remnant of the retreating primitive streak. The mesenchymal cells in the dorsal portion of the tail bud undergo condensation and epithelialization. This forms a rod like condensation of mesenchymal cells. A central canal forms within the rod by cavitation. The central canal then becomes continuous with the one formed during primary neurulation and thereby forms the secondary neural tube (154; 155). In humans, neural tube closure (NTC) was originally hypothesized to initiate at five closure sites (156). More recent clinical examinations have only identified two to three of these closure initiation sites; where closure I contributes to the posterior neuropore, while closures II and III complete closure at the anterior neuropore (157; 158). The pathogenesis of NTDs is often believed to involve a failure in cellular proliferation, alterations in the shape of the developing neuroectoderm, or negative changes in vascular development supporting these cells (159). The causes of these alterations is thought to be multifactorial, with contributions from both biological and environmental aspects (160).

In order to understand the factors involved in a dynamic biological process, such as neural tube closure (NTC), the interaction between DNA sequences (genomics), RNA

levels (functional genomics), and expressed proteins (functional proteomics) needs to be carefully examined (161). When properly studied, teratogenesis in an organism can be approached at each of these organizational levels and may provide susceptible genotypes, gene expression classifiers, and diagnostic protein profiles.

At present, studies are being conducted at each level of biological organization in order to better understand NTC and the subsequent development of NTDs. Unfortunately, the biological components contributing to the population burden of NTDs have been difficult to elucidate. To date, no one single major gene has been implicated as a direct causal agent for these defects (162), a result common in genetic association studies on complex diseases (163). Additionally, genetic linkage studies to identify critical polymorphisms are difficult to accomplish due to a shortage of multiple affected families. Despite this difficulty, by utilizing experimental animal models, scientists are able to explore the expression of those genes that are involved in biochemical transport and metabolism thought to be critical to normal NTC. Biochemical factors such as folic acid appear to be the greatest modifiers of NTD risk in the human population, and may play an important role in other serious structural malformations (164; 165). A reciprocal interaction also exists between the genome and nutritional elements, insofar as the influence of gene haplotypes can affect the metabolic efficiency of the organism (166).

Considering the cellular processes whose disrupted function can lead to NTDs, candidate genes that have been the focus of teratogenic investigations are often those involved in folate biochemistry, cell proliferation, apoptosis, cellular adhesion, changes in vascular development, *de-novo* methylation, neural induction or neural pattern

formation (159; 167). Furthermore, in support of these gene categories being causal of teratogenesis, over 80 genes with a diversity of functions have been associated with neural tube defects when they are inactivated by gene targeting (167). These genes include transcription factors, transcriptional co-activators, cell surface/membrane receptors, signaling proteins and signal transducers (168-172).

Role of Folic Acid in Prevention of Neural Tube Defects

Folate deficiency has been associated with the occurrence of selected birth defects, and a considerable number of human studies have demonstrated the protective role of maternal folic acid supplementation (5; 173). These studies demonstrated that maternal folic acid supplementation of at least 0.4 mg/day reduces the incidence of NTDs by up to 70%. A recently published study by Rothenberg and colleagues (2004) demonstrated that serum from women whom had a pregnancy complicated by an NTD contained autoantibodies that bound to folate receptors and blocked the cellular uptake of folate (174). Rothenberg suggested that given the antibody driven uptake changes, folate supplementation allows alternative cellular entry or increases competition with the antibody's binding of folate receptors in order to restore folate homeostasis. These experiments parallel what has been observed in the *Folbp1* knockout mouse model, and clearly demonstrate the importance of intercellular uptake of folate (175; 176). In contrast to these observations, the cellular mechanism(s) governing why perturbations of folate uptake are teratogenic remain unknown.

Folate Biochemistry and Folate Receptors

Folic acid is a biologically inactive water-soluble B vitamin that is transformed to its bioactive forms *in vivo* (177). Folic acid is known to contribute to three metabolic pathways: remethylation, the folate cycle, and transsulfuration. In one-carbon metabolism, folic acid donates methyl groups via the remethylation pathway for the methylation of nucleic acids, lipids, neurotransmitters, and also for the post-translation modifications of numerous other proteins. The methylation reactions are catalyzed by methyltransferase (MT) enzymes, which require S-adenosylmethionine (SAM) as the specific methyl donor. The metabolic process that generates SAM occurs primarily via the remethylation pathway, where the conversion of homocysteine to methionine requires the transfer of a methyl group from 5-methyl-tetrahydrofolate, the primary bioactive form of folate. Methionine is then converted to SAM by methionine adenosyltransferase and ATP. After donating its methyl group to acceptor molecules, SAM is converted to S-adenosylhomocysteine (SAH) (178).

Folic acid is also biologically essential for the synthesis of nucleic acids. Clinically, low folate can induce megaloblastic anemia with an etiology stemming directly from impaired DNA synthesis. In the *de novo* synthesis of purines, carbons from the folate one-carbon pool form parts of the purine ring. The folate metabolite 5, 10-methenyl tetrahydrofolate is used in the synthesis of the five-atom ring (position 8), and 10-formyl tetrahydrofolate is used in the synthesis of the six-atom ring (position 2). The final product is inosinic acid (IMP), which is used to produce AMP or GMP for RNA synthesis or as DNA precursors. Carbons from the folate one-carbon pool are also

involved in *de novo* pyrimidine synthesis. The enzyme thymidylate synthase catalyzes the transfer of 5,10-methylene tetrahydrofolate to the pyrmidine ring of dUMP. This reaction produces dTMP, which is the precursor of the dTTP used in DNA synthesis (178). Transport of folates into cells is principally accomplished by way of the reduced folate carrier (RFC) and the folate receptors (FR), also known as folate binding proteins (Folbps) in mice. There is one definitive gene for RFC in humans and mice. There are at least four human folate receptor isoforms (FR-α, FR-β, FR-γ, FR-δ) and three murine receptors (Folbp1, Folbp2, Folbp4) that are currently known. They are all considered orthologs respectively, but FR-γ / Folbp3 has not been observed in mice. The folate receptor isoforms have been shown to have tissue-specific and cell-specific expression patterns (179-181). Functionally, the differences in the protein products of FR-α and FR-β are observed in binding of folate, where FR-α has a 50-100 fold greater affinity depending on the folate substrate. The folate receptor isoforms are externally bound to the plasma membrane by glycosyl-phosphatidylinositol (GPI) anchors; however, a high-

(A)

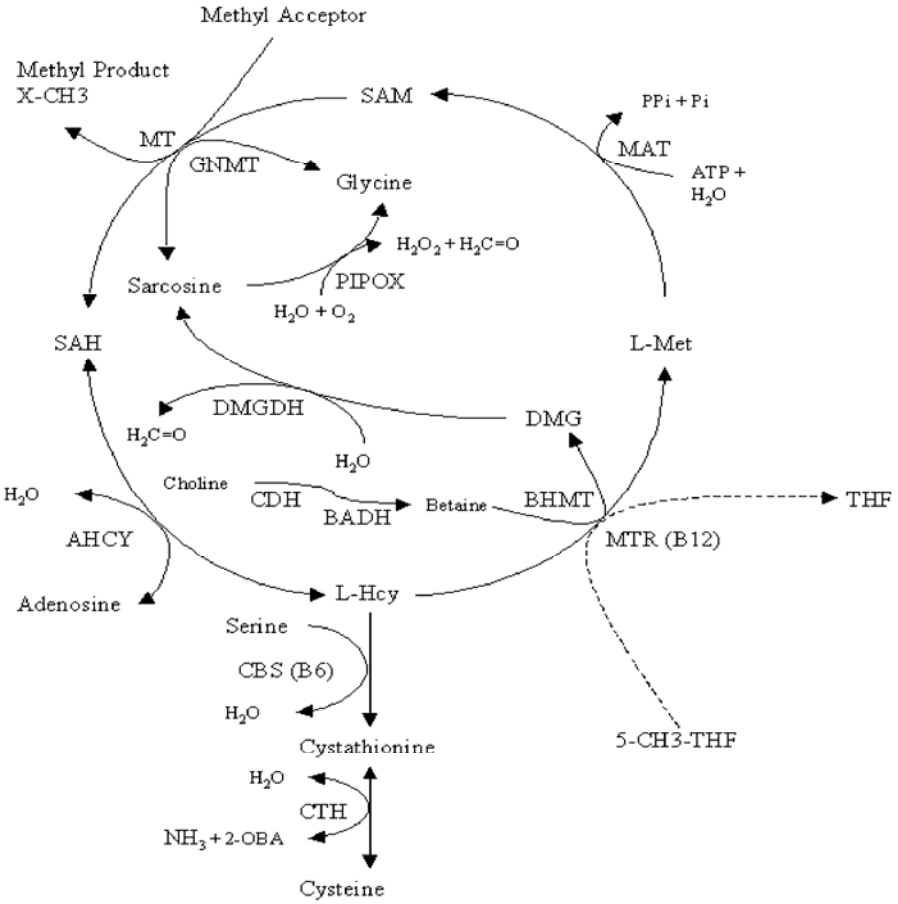

Figure 2.1. Genes and metabolites of folate cycle, remethylation and transsulfuration. Pathway Information was extracted from KEGG: Kyoto Encyclopedia of Genes and Genomes at http://www.genome.jp/kegg/ and revised with reference entries at NCBI: National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov/. Folates include: DHF – dihydrofolate, THF – tetrahydrofolate, 5,10-CH2-THF - 5,10-methylene-tetrahydrofolic acid, 5,10-CH=THF - 5,10-methenyltetrahydrofolate, 5-CH3-THF - 5-methyltetrahydrofolate, 10-HCO=THF - 10-Formyltetrahydrofolate. (A). Remethylation and transsulfuration genes (Enzyme Commission Number). GNMT - glycine N-methyltransferase (2.1.1.20), PIPOX - pipecolic acid oxidase / peroxisomal sarcosine oxidase (1.5.3.1), DMGDH - dimethylglycine dehydrogenase (1.5.99.2), BHMT - betaine-homocysteine methyltransferase (2.1.1.5), CDH / CHDH - choline dehydrogenase (1.1.99.1), BADH / ALDH9A1 - betaine-aldehyde dehydrogenase, aldehyde dehydrogenases E3 (1.2.1.8), MTR / MS - 5-methyltetrahydrofolate-homocysteine methyltransferase / methionine synthase (2.1.1.13), MAT1A, MAT2A - methionine adenosyltransferase 1, 2, 3 (2.5.1.6), AHCY, AHCYL1, KIAA0828 - S-adenosylhomocysteine hydrolase, S-adenosylhomocysteine hydrolase-like 1, KIAA0828 protein (3.3.1.1),CBS - cystathionine-beta-synthase (4.2.1.22), CTH - cystathionase (4.4.1.1)

(B)

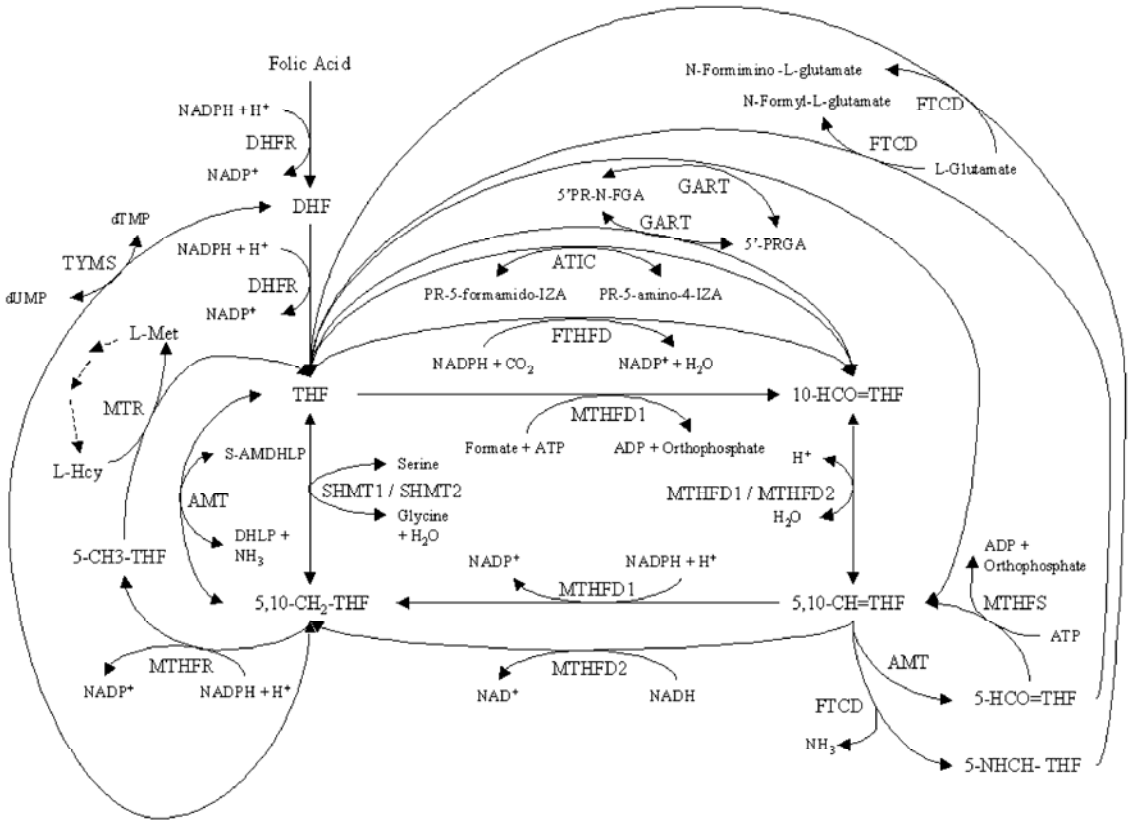

Figure 2.1 Continued, (B). Folate Cycle Genes (Enzyme Commission Number).
DHFR - dihydrofolate reductase (1.5.1.3)
ATIC - 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (2.1.2.3)
GART / PRGS / PAIS - trifunctional glycinamide phosphoribosyltransferase (2.1.2.2)
FTHFD - 10-formyltetrahydrofolate dehydrogenase (1.5.1.6)
FTCD - formiminotransferase cyclodeaminase (2.1.2.5)
AMT / GCST - aminomethyltransferase / glycine cleavage system T protein (2.1.2.10)
SHMT1, SHMT2 - serine hydroxymethyltransferase 1, 2 (2.1.2.1)
MTR / MS - 5-methyltetrahydrofolate-homocysteine S-methyltransferase (2.1.1.13)
TYMS / TMS / TS - thymidylate synthetase (2.1.1.45)
MTHFD1 / MTHFC / MTHFD - methylenetetrahydrofolate dehydrogenase (NADP+ dependent) / methenyltetrahydrofolate cyclohydrolase / formyltetrahydrofolate synthetase (1.5.1.5)
MTHFD2 - methylene tetrahydrofolate dehydrogenase (NAD+ dependent) / methenyltetrahydrofolate cyclohydrolase (3.5.4.9)
MTHFS - 5,10-methenyltetrahydrofolate synthetase / 5-formyltetrahydrofolate cyclo-ligase (6.3.3.2)
MTHFR - 5,10-methylenetetrahydrofolate reductase (NADPH) (1.5.1.20)
Cofactors. S-AMDHLP - S-aminomethyldihydrolipoylprotein, DHLP – dihydrolipoylprotein, PR-5-amino-4-IZA - 1-(5'-phosphoribosyl)-5-amino-4-imidazolecarboxamide, PR-5-formamido-IZA - 1-(5'-phosphoribosyl)-5-formamido-4-imidazolecarboxamide, 5'-PRGA - 5'-phosphoribosylglycinamide 5'PR-N-FGA-5'-phosphoribosyl-N-formylglycinamide

affinity folate receptor has also been observed unbound without GPI modifications in plasma (182). The unbound form of folate receptor increases during folate deficiency and promotes the stabilization of tetrahydrofolate (183). The affinity and sequencing analysis of the unbound form indicates it as a variant of FR-α. Accordingly, FR-α and Folbp1 are primarily found in the plasma, placenta, choroid plexus, and the brush border membranes in kidney (184; 185). In the developing mouse embryo, *Folbp1* has been observed to be highly expressed in the neural folds, yolk sac, and neural tube (186).

There are over 25 proteins involved in the remethylation, folate cycle, and transsulfuration pathways (Figure 2.1). Several of the corresponding genes have been examined as risk factors for NTDs, and fewer still have been associated with risk rates for NTDs (164; 187; 188). In this later group, the MTHFR 677C→T mutation first emerged as a possible genetic risk factor for NTDs (189-191). A second mutation in the MTHFR gene (1298A→C) has also been associated with NTDs (192; 193). NCBI's SNP database www.ncbi.nlm.nih.gov/SNP/ presently shows 88 single nucleotide polymorphisms (SNPs) in the region; 14 of which are mutations (6 nonsynonymous) within the MTHFR gene itself (LocusID:4524). Of these 6 nonsynonymous mutations, only the 677C→T and 1298A→C have been associated with a minor risk modification for NTDs.

Mutations in genes for cellular uptake of folate receptors has been closely related to NTD rates in animal models, but thus far, there has been no evidence for such mutation being related to increased risk for NTDs in humans (194). In this regard, NCBI reports there are presently 22 SNPs in region of FR-α (LocusID:2348), of which 2 are

nonsynonymous (107T→C, 505G→C), but neither have been implicated in modifying NTD rates. On the other hand, a highly common mutation in RFC (80A→G) has recently been tested for modifying NTD risk rates (195). Additional studies demonstrate that this mutation may also interact with low folate and MTHFR mutations to increase the risk of NTDs (196; 197).

Environmental factors such as maternal vitamin use in the presence of gene variants directly involved in folic acid metabolism and uptake comprise a large part of gene-environment (GE) associations considered in NTD studies. In this context, the MTHFR 677C→T mutation is related to decreased MTHFR activity, low plasma folate, and high plasma homocysteine (198-200). Penetrance of this polymorphism is suggested to be modified by dietary and supplemental folate (198; 200; 201) and has highly variable allelic frequencies across ethnic backgrounds (202). Likewise, variations at the infant RFC (80A→G) locus appear to modify the effects of folate supplementation on NTDs. Individuals that are homozygous mutant (G80/G80) for RFC confer a modest decrease in risk of NTDs when supplemented with folic acid, compared to the non-folate supplemented pregnancies (196). Risk of NTDs may also be lower for this genotype when red blood cell (RBC) folate is low relative to individuals having normal RBC folate levels (203).

Vitamin B12

In 1964, Dorothy Hodgkin identified the structure of vitamin B12 (204), which had already gained medical importance in the treatment of pernicious anemia (205; 206).

This condition begins with megaloblastic anemia but unlike megaloblastic anemia induced by folate deficiency, the B12 symptoms progress into degeneration of the nervous system. Pernicious anemia is now recognized as a disease of the stomach, where intrinsic factor has been shown to actively bind and allow B12 absorption through the small intestine via the luminal membranes of ileal cell (207; 208). Data indicates pernicious anemia can result from insufficient secretion of intrinsic factor or competitive inhibition of intrinsic factor by autoantibodies that block B12 binding and absorption in the small intestine (209; 210).

Vitamin B12 was later identified as a potential risk factor for NTDs, but there has also been considerable debate with this observation. Several studies show no differences between serum levels of B12 or folate in NTD case versus control mothers (211-213), while other studies show lower serum B12 levels in NTD cases (214-217). This controversy has been the subject of a recent review where it was concluded that there are moderate associations between low maternal B12 and an increased risk of NTDs (218). Other studies also indicate that B12 concentrations appear modified internally across the placenta, where a lower B12 status may or may not be present in serum but is significantly lower in the amniotic fluid of NTD case versus control mothers (219-223). Additionally, Afman et al. (2002) studied 5 SNPs in the coding region of the Transcobalamin 2 (TCN2) gene, but none of the SNPs were associated with a significantly increased risk for NTDs. Three of the TCN2 SNPs affected the transcobalamin concentration, but these variants only partially accounted for the reduced proportion of vitamin B12 binding observed (224).

Extending the vitamin B12 hypothesis, studies have also focused on folate and vitamin B12 related genes such as methionine synthase (MTR) and MTR reductase (MTRR); these studies indicate that MTRR-dependent reduction of B12 may increase the risk of spina bifida (225; 226). Futhermore, studies on folate and vitamin B12 enzymes have expanded to include gene-gene interactions or epistasis between MTHFR 677C→T, MTHFR 1298 A→C, MTRR 66A→G, serine hydroxymethyltransferase (SHMT 1420C→T), cystathionine beta-synthase (CßS 844ins68), glutamate carboxypeptidase II (GCPII 1561C→T), and RFC 80G→A (203; 226; 227). Collectively, the genes involved in folate biochemistry and their respective polymorphisms appear to only produce small risk increases. This has introduced the idea that cumulative environmental conditions act on individuals with specific genetic predispositions, leading to threshold infringement, after which a NTD is produced (152; 228).

Due to the different results of B12 status between sera and amnion in the aforementioned studies and the recent study of increased rates of NTD cases among women with folate receptor autoantibodies (174), we suggest that autoantibodies to intrinsic factor, transcobalamin 1 (TCN1) and 2, all of which increase the risk for pernicious anemia, may also increase the risk for NTDs. In light of intrinsic factor, TCN1 and 2 also being found in the amnion (229), this hypothesis may also explain the observed differences in concentration of vitamin B12 in different tissues.

Human Teratogens

Teratogens associated with the induction of NTDs are shown in Table 2.1. There are an increasing number of investigations into suspected teratogens but relatively few have been or can be confirmed in population-based studies. Specifically, teratogens that are not prescribed medically or are environmental contaminants may be associated with NTDs, but the data are more difficult to assess in the later environmental groups due to non-medical control of usage and exposure. In order to elucidate the mechanisms of known teratogens, or further characterize suspected teratogens, researchers may also utilize animal models.

Testing Teratogens in Models

The use of animal models to enhance our basic understanding of the regulation of events involved in development has led to the identification of various candidate genes for susceptibility to teratogenesis in humans. Model systems confirm that there are multiple developmental pathways and multiple genes involved in organogenesis that result in the same clinical malformations. They also demonstrate that interactions of environmental and genetic factors contribute directly to these aberrant phenotypes. Chemical interactions may be protective if the compound reduces the risk for birth defects (*e.g.* folic acid), or teratogenic if the substance increases the prevalence of abnormalities (*e.g.* valproate). Clearly, gene-chemical interactions, gene-gene interactions, gene-environment interactions, and the maternal environment affecting fetal development all contribute to the ultimate phenotype, making downstream analysis incredibly complex.

When utilizing a model species in order to elucidate the mechanism of NTD teratogenesis, the hypothesis is tested upon the model itself, and extrapolating these findings to human populations may not always be valid for risk assessment (230). Another such limitation involved in experimental animal testing is the use of inbred strains. The inbred strain is essential to many investigations, yet extrapolation of the results of a near homologous population of experimental animals to a heterogeneous population of humans can lead to unanticipated error (231). In addition, using animal bioassays requires a significant time and financial commitment in order to properly perform the testing. Challenges to using animal models to understand teratogenesis involves a legacy of unresolved issues over the relevance of toxicity data in risk assessment, the incorporation of novel technology platforms into hazard evaluation, and the societal challenge that demands safer products that are tested on experimental animals in face of disapproval and threat from various and diverse sections of society (232).

In order to properly assess human exposure levels, it is essential that the data from animal models detect the effects of teratogens. Most studies encompass the use of several species for animal testing under different exposure conditions. Exposures include the assessment of maternal and embryo toxicity in order to establish appropriate dosages.

Table 2.1. Pharmaceutical teratogens.

Category	Drugs		Use	Teratogenic Mechanism
Anticonvulsants	Carbamazepine Clonazepam Ethosuximide Phenobarbital	Valproic Acid Phenytoin Primidone	Anticonvulsants are used to prevent and minimize seziures. Therapeutic action - several are suspected including effects on ion channels, active transport, and membrane stabilization.	Unknown - may include inhibition of folate metabolism and/or inhibition of histone deacetylase.
Folate Acid Antagonists (FAAs)	Aminopterin Methotrexate Triamteren	Trimethoprim Pyrimethamine	FAAs can be used in the treatment of cancers or infections. Therapeuric action - via substrate inhibition of enzymes involved in folate metabolism, which in turn stalls cellular proliferation.	Unknown - may include nucleoside synthesis inhibition and/or inhibition of methylation reactions.
Diuretics	Unspecified		Diuretics increase the discharge of urine. Therapeutic action- they are a diverse group of compounds affecting various hormones regulating urine production by the kidneys. Some diuretics are sulfonamide derivatives.	Unknown
Antihistamines	Unspecified		Antihistamines of this type are used to ameliorate the symptoms of allergic response. Therapeutic action- most commonly used are the first generation H1-antagonists, which are also potent anticholinergic agents.	Unknown
Sulfonamides	Sulfamethoxazole	Sulfamethazine	Sulfonamides are a sulfur dioxide and nitrogen moiety linked to a benzene ring. Antibacterials are made by the addition of an arylamine side chain. Therapeutic action- they are competitive inhibitors of PABA, a substrate important in bacterial folate production. The addition of trimethoprim, which inhibits bacterial dihydrofolate reductase, creates synergistic preparations.	Unknown - may include nucleoside synthesis inhibition and/or inhibition of methylation reactions.

When the data from several species is clearly dose-related in causing neural tube defects, extrapolation to humans is considered more reliable. The problem arises with this method when a malformation is observed in only select species. This may signal a potential hazard for humans, but ultimately the data cannot be directly extrapolated. Therefore, when these effects are observed in a test species, adverse outcomes in human pregnancy must then be established based upon more mechanistic approaches (233).

Correlating Mouse and Human Models

It is unfortunate but very few of the mouse models of NTDs mirror the human situation with respect to non-syndromic cases. Such isolated NTDs occur with incomplete penetrance, have an unclear transmission pattern, and great genetic complexity. Although variable low penetrance is found in most of the mutant models, only a few strains show the complex inheritance patterns that are indicative of human non-syndromic NTDs (234). SELH/Bc is one of the few mouse models that do show non-syndromic NTDs with distinctive genetic complexity. SELH/Bc embryos are unable to complete normal closure at closure site II, with 20 percent developing exencephaly. However, most embryos develop into normal adults despite their abnormal embryology, as they compensate by extension of closure III to fully complete NTC (234). For further information on mouse models with NTDs, there are a handful of recently published concise reviews (167; 234).

Pharmaceutical Induction of NTD

Several pharmaceutical compounds have been associated with an increase in risk for NTDs. Specifically, there is a direct relationship with antiepileptic drugs (AEDs) and the development of NTDs (235). AEDs are a diverse class of pharmaceuticals with chemical compositions and structures that vary considerably. In spite of these differences, in utero exposure during the first and second months of pregnancy to widely prescribed AEDs such as valproate and carbamazepine are associated with a 10-20 fold increase in the frequency for NTDs in humans (236-239). Exposure to multiple AEDs, as is often the case in the management of seizures in epileptic patients, can elevate the frequency of NTDs even further (240; 241). This increasing risk is reflected in data that strongly supports the use of monotherapy to control epilepsy during pregnancy (242).

Valproate (VPA, Depekane, Abbott Laboratories) is by far the most highly studied compound for teratogenicity of AEDs (243). Numerous animal models have demonstrated a dose-response to valproate induction of NTDs (244-246). The mechanism of valproate teratogenicity is still unknown, but considerable steps are being made in this direction. VPA and other AEDs are folic acid antagonists due to their ability to inhibit dihydrofolate reductase, decrease folate absorption, or increase folate degradation (237; 247). Genetic differences in NTD susceptibility to valproate have also been demonstrated in murine inbred strains (248). These differences have been utilized in breeding schemes that suggest genetic imprinting in VPA teratogenicity (249; 250), and have also been used to identify a region of the murine genome strongly linked to susceptibility of VPA-induced NTDs (251). Recentlly, VPA has also been characterized

as an inhibitor of histone deacetylase (HDAC) (252; 253). Furthermore, it was demonstrated that VPA analogs without the ability to inhibit HDAC were not teratogenic in *Xenopus* (252). Collectively, these studies provide new insight into the etiology of NTDs and exemplify the complexity of pharmaceutical induced teratogenicity. Futhermore, we suggest that collectively, this data is consistent with the threshold model for the induction of NTDs and other complex traits.

In addition to AEDs, associations with NTDs have also been found with diuretics, antihistamines, and sulphonamides (254). However, another study was unable to reproduce these results (255). Ultimately, the results on non-AED drugs are still equivocal, and will require additional studies for prudent characterization with respect to their ability to induce NTDs in the human population.

Maternal Obesity and Diabetes

While a complete consensus concerning the risk of maternal obesity and diabetes on NTDs has not been reached, studies suggest that blood glucose concentrations can be considered as an NTD risk factor. Maternal diabetes has long been associated with NTD risk (128-130), while periconceptional glycemic control has been associated with a reduction in risk (131-133). Maternal hyperinsulinemia and hyperglycemia are also suggested to be putative risk factors for NTD affected pregnancies (256; 257). Mothers, particularly obese mothers, having a higher sugar intake are at a significantly increased risk for having an NTD-affected pregnancy (258-260). This is particularly relevant when considering research correlating high body mass index (BMI) with elevated glucose

concentrations in non-diabetic mothers (261). A recent review also presents a discussion of these risk factors (262).

A retrospective epidemiologic study demonstrated a strong association between risk of an NTD affected pregnancy and maternal glycemic index among nondiabetic pregnancies (260; 262). Involving nearly 1000 participants who were almost evenly divided between cases and controls, the risk of an NTD-pregnancy was not significantly elevated in relationship to glucose or fructose intake, although there was a modest correlation for sucrose intake. This effect was consistent for all NTDs when analysis was adjusted for energy intake. In addition, there was a strong interaction between maternal pregnancy weight, maternal nonpregnancy weight, and glycemic index in NTD risk. When adjusted for folate status, maternal education, race or ethnicity, and total energy intakes, the elevated NTD risk was limited to higher glycemic index values in women with a BMI greater than 29. While any retrospective study is subject to random variation and recall bias, and glycemic intake of foods in combination with activity level was used as a proxy for serum glucose concentrations, it is unlikely that these would have resulted in the quadrupling, or more, of NTD risk for the nondiabetic obese women consuming foods with a high glycemic index that was observed.

Elevated maternal serum glucose concentrations during pregnancy may directly affect NTC. The developing human embryo lacks pancreatic function until after the 7th week of pregnancy, and is therefore subject to maternal serum glucose concentrations. This could expose the developing conceptus to harmful concentrations of glucose during NTC (261). In rat embryos, elevated glucose concentrations are known to induce

oxidative stress and inositol depletion (263; 264). It has also been shown that inositol supplementation is protective against folate-resistant posterior-NTDs in mice (265), but it is not known whether there is a similar inositol protection in humans.

Animal models have suggested that the *Pax* gene family may modify risk of NTDs in diabetic pregnancies (266; 267). A mouse model of diabetic pregnancy demonstrates an increase in NTDs accompanies a significant decrease in the transcription factor, *Pax-3* gene expression (266). This potential interaction has not been substantiated in human studies; however, due to small sample sizes it is possible the existing studies in the literature lacked the power required to detect such an interaction (268-272).

Maternal Hyperthermia

The range of acceptable mammalian maternal core temperatures is from 39.5°C to 35.5°C, while the range between hyperthermia- and hypothermia- induced coma is 41°C to 33°C. Embryonic development, and NTC in particular, has proven especially refractory to hyperthermia. Marshall Edwards first demonstrated that a 3°-5°C elevations during the initial stages of neurulation were teratogenic in laboratory rodents, and the literature suggests that even small elevations in the core temperature of 1.5-2.4°C are detrimental to mammalian neurulation (273; 274). Knowing that developmental defects were observed in animal species (guinea pigs, mice, rats, chicks, and monkeys) exposed to a hyperthermic insult of 2°C or more above normal body temperature, it is reasonable to suspect that human embryos are similarly susceptible (274).

With the advent of hot tubs as a recreational device, anecdotal clinical reports, followed by retrospective epidemiologic studies brought this potential developmental hazard to the attention of the medical community (275). In humans, anencephaly, meningomyeloceles, and encephaloceles have all been associated with an elevated maternal core temperature, increasing risk as it reaches 38.9°C, and especially as it exceeds 40°C. (276-283). These elevations in temperature can be secondary to febrile illness, occupational exposures, or recreational activities. In a comparative study involving 23,491 women of hyperthermic exposure limited to sauna, hot tub, fever, or electric blanket, it was found that early hyperthermic exposure roughly doubled the risk of an NTD-complicated pregnancy, while exposure to more than one of these factors increased risk 6-fold (283).

In humans, the greatest hyperthermic risk is generally considered to be from maternal febrile illness. In a large retrospective population-based case-control study of fetuses and liveborn infants with NTDs, maternal fever or febrile illness in the first trimester was associated with a two-fold increased risk for an NTD pregnancy (284). However, the possibility that the increased risk was due to reporting bias cannot be completely discounted. Additionally, the etiology of these NTD-affected pregnancies is difficult to disentangle from maternal hyperthermia and the illness itself (285). In support of hyperthermia proper being a risk factor, data from experimental animals demonstrate the increased NTD incidence without pathogenic exposures.

Edwards first showed the hyperthermia-NTD relationship by exposing guinea pigs to hyperthermic insult immediately prior to neurulation. The resulting progeny

presented with defects of the abdominal wall, and hypoplastic digits, but most of the anomalies were confined to the neural tube, brain, and skeleton. In hamsters that were similarly exposed, NTDs were the most common defect (286). Additionally, hyperthermic insult resulting from radiation exposure has been proven to be an effective method of inducing NTDs in rats (278; 283; 285; 287).

Evidence in animal models suggests that induction of NTDs through elevation of the maternal core temperature is an embryonic, not a maternal effect. This has been investigated by completely removing the embryo from maternal influence and exposing rat embryonic explants during the onset of NTC to mild hyperthermic insult which proved to be teratogenic. Embryos thus exposed displayed head and somite malformations, with nearly half of the exposed embryos being microcephalic (288). Temperature and duration of exposure act together in a dose-response relationship to induce NTDs in experimental animals (289). This provides an explanation for the failure of saunas to induce elevated rates of NTDs in Finnish women, who regularly indulge in saunas while pregnant (290). Consistent with this explanation, subjects in another study could not tolerate hot tub exposure long enough to elevate their core temperature to 38.9°C (291).Susceptibility to hyperthermia induced NTDs also appears to have a strong genetic component. This has been demonstrated in laboratory mice with pronounced differences in response frequencies of hyperthermia induced NTD. Susceptibility for mice has been shown to range from completely resistant (DBA/2J) to highly sensitive (SWV/Fnn), where the latter group displayed 44.3% exencephaly following exposure (292). Data from this study also suggest that only a relatively few genes are responsible

for this variation in NTD susceptibility and that the embryonic genotype is the major modifier of this susceptibility.

Fumonisin B_1

Fumonisin B1 (FB_1) has been identified as a potential teratogen and risk factor for NTDs in populations consuming large amounts of fumonisin-contaminated corn products (293-297). Fumonisins are a family of mycotoxins produced by *Fusarium verticilloides*, commonly found as a contaminant of corn. The major isoform, fumonisin B_1, has been recently recognized as a teratogen (298), and previous studies also showed it causes liver and kidney cancer in rats and mice (Gelderblom, NTP) and is a suspected human carcinogen (299).

Receptor-mediated folate uptake was found to be reduced by up to 50% in Caco-2 cells pretreated with fumonisin (300). Due to Fumonisin's structural resemblance to sphingoid bases, it disrupts sphingolipid biosynthesis by inhibiting ceramide synthesis (301-303). This pathway is required for GPI-anchoring, and fumonisin thereby disrupts the GPI proteins such as folate receptor. This in turn disrupts the receptor's endocytic recycling (304) and the amount of the receptor available for substrate transport (294).

In several animal models, maternal oral fumonisin exposures have been largely unsuccessful in producing NTD phenotypes (305-307), but fumonisin does produce NTDs in developing murine explants in addition to significant growth retardation (308). Explants also displayed dose-dependent exencephaly that could be partially mitigated by the addition of folate to the growth medium (298). Murine studies also showed that

maternal IP dosing with fumonisin during neurulation induced exencephaly in Lm/Bc/Fnn mice which was reduced by folate supplementation (Gelineau-van Waes and Maddox, personal communication). Fumonisin's role in the disruption of folate transport and lipid rafts was further supported by rescue of fumonisin-exposed dams with gangliosides (10 mg/kg) the day before, during, and the day following exposure (294).

Other Risk Modifiers for NTDs

Many environmental contaminants have been suspected of being human teratogens. These contaminants include organic solvents (309; 310), chlorination disinfection by-products in drinking water (311-314), and pesticides (310). These studies rarely associate exposure with a greater than a two-fold increased risk of an NTD-affected pregnancy. Metals are another area of concern with respect to NTD teratogenesis. Periconceptional exposure to cadmium (315) and lithium carbonate (316) has been associated with NTDs in animal models, but human studies have shown no increased NTD risk from exposure to tap water contaminated with a variety of metals including: lead, calcium, magnesium, copper, lithium, zinc, nickel, selenium, mercury, chromium, silver, cobalt, cadmium, and molybdenum (317; 318).

There has also been data that indicates a relationship between folate and iron, in that the metal can modulate the availability of folate, leading to symptoms of folate deficiency despite adequate folate intake and extracellular folate concentrations (319). Investigations shed light on this relationship by demonstrating that the homocysteine remethylation pathway is regulated in part by serine hydroxymethyltransferase (SHMT),

whereby SHMT's gene expression is regulated by the heavy-chain subunit of ferritin (320). It has been suggested that lead may induce NTDs by reducing bioavailability of dietary zinc (321-324).

Other exposures of interest which require additional research in order to further establish their importance as human teratogens include: maternal diarrhea (325), maternal emotional stress (326), electromagnetic fields (327; 328), proximity to hazardous waste sites (329; 330), and pesticides (331).

While there are many agents suspected of possessing a teratogenic potential, there are relatively few confirmed environmental teratogens. This may be due to several factors, including: 1) laboratory studies performed on mice generally utilize doses of teratogens that are much higher than that which humans would generally be expected to encounter; 2) environmental exposure is much more difficult to quantify in retrospective studies involving humans than in mice kept under laboratory conditions; and 3) human susceptibility to these environmental teratogens may hinge on complex interactions of genetic susceptibility, environmental exposure to teratogens, and availability of protective agents, such as dietary folate.

DISCUSSION

The interactions between environmental factors and target genes are critically important to the regulation of developmental pathways contributing to the formation of an embryo. Evidence from mouse models suggest that the multiple genes/proteins involved may be contributing in an additive or multiplicative fashion; this can set the embryo on a path of

abnormal development should the regulation of these genes/proteins be perturbed by exogenous factors. A long-standing teratologic concept is that an organism of a given genetic sensitivity may be subject to multiple environmental factors that are potentially teratogenic; yet remain below the threshold for expression of an abnormal phenotype. The abnormal phenotype may present at a time when these multiple factors reach some threshold at which normal development can no longer proceed. Such additive factors ultimately contribute to a final phenotypic expression of a neural tube defect (152; 228). Although it is highly unlikely that any single gene is responsible for the risk of developing birth defects in the majority of human clinical cases, animal models have efficiently proven their worth in identifying candidate genes for further investigations in human molecular epidemiologic studies. However, it is important to temper any conclusions with the realization that modifier genes, stochastic processes, distinct targets for specific isoforms and functional redundancies all may lead to different phenotypic outcomes (332). Additionally, when studying NTDs or NTC, it is essential to view them as dynamic biological processes. In these processes, the presence of teratogens or absence of essential biological factors can have population and individual-based influences according to ethnicities or genetic backgrounds.

Studies of complex disease etiology for gene-gene and gene-environment interactions have expanded in methodology and matured in analyses, yet the existing body of literature is still constrained by limited sample sizes. Support for genetic variants and environmental exposures as independent risk factors has proven difficult, and evidence for gene-gene and gene-environment interactions is often equivocal. Befitting its complexity, methods to establish the genetic and biochemical underpinnings of NTDs must continue to evolve. Greater focus should be placed on increasing sample size, evaluation of multiple loci and effect modification, and determination of the most relevant phenotype (e.g. having a child/pregnancy affected by a NTD or having a NTD) and genotype (maternal/embryonic) (333). These highlights are presented in anticipation of the increasing number of studies by investigators into the etiology of NTDs and the growing level of understanding proffered to the scientific community by these studies.

CHAPTER III

ARSENIC-INDUCED GENE EXPRESSION CHANGES IN THE NEURAL TUBE OF FOLATE TRANSPORT DEFECTIVE MOUSE EMBRYOS*

OVERVIEW

Arsenic injected intraperitoneally (ip) during early organogenesis to small pregnant laboratory rodents (mouse, rat, and hamster) induces several congenital defects in the progeny. Among those abnormalities consistently and predominantly observed are exencephaly and encephalocele. These severe defects of the central nervous system originate from a corrupted process of neurulation and are better known as neural tube defects (NTDs). In order to understand the mechanism of arsenate-induced NTDs, we designed studies in which highly sensitive Folr2 nullizygous mice were injected intraperitoneally with sodium arsenate at the beginning of the neural tube formation process. This specific knockout mouse and the arsenic exposure conditions were chosen as they were known to provide a high incidence of exencephaly in exposed embryos. We have applied gene expression technology to the anterior neural tube. This allowed us to study arsenic induced changes in patterns of gene expression that may contribute to the development of neural tube defects in these mice. Using extensive data analysis approaches including hierarchical clustering and gene ontology analysis, we identified several candidate genes as well as important ontology groups that may be responsible for

* Reprinted with permission from "Arsenic-induced gene expression changes in the neural tube of folate transport defective mouse embryos" by Wlodarczyk B.J., Cabrera R.M., Hill D.S., Bozinov D., Zhu H., Finnell R.H., 2007. *Neurotoxicology*, 27:547-57, Copyright (2007) by Elsevier.

arsenic's teratogenicity. Changes in the expression of several genes in response to arsenic treatment in our model had previously been demonstrated by other investigators to also induce NTDs in murine model systems. These include: *engrailed 1 (En-1)*, *platelet derived growth factor receptor alpha (Pdgfrα)* and *ephrinA7 (EphA7)*. We also found several gene ontology groups that could be implicated in arsenic's underlying teratogenicity: morphogenesis, oxidative phosporylation, redox response, and regulation of I-kappaB kinase/NF-kappaB cascade. Additionally, we revealed new target genes which may be responsible for arsenic disrupted oxidative phosphorylation.

INTRODUCTION

Arsenic posses "the most significant potential threat to human health due to its known or suspected toxicity and potential for human exposure" of the 275 most hazardous substances found in the environment (334). This list, created by the Agency for Toxic Substances and Disease Registry, is based on the toxicity, and potential for exposure at any one of the National Priorities List, or "Superfund" sites. While arsenic is ubiquitous in the environment and is found in varying concentrations in rock, soil, and water, anthropogenic sources of arsenic including metalworking industries (including the smelting of lead, copper, and nickel), coal combustion, semiconductors and pesticides production, results in tons of arsenic being released into the environment every year. According to the United States Environmental Protection Agency's (USEPA) Toxic Release Inventory (TRI), in the year 2003, 289,629,256 pounds of arsenic or arsenic compounds were reported released as a consequence of "Total On- and Off-site Disposal

or Other Releases". This is not a comprehensive number, as only a portion of relevant facilities are required to report to the TRI. The consequence is a greatly increased circulation of potentially hazardous compounds into the environment. Increased human exposure to arsenic compounds carries with it unknown health risks for future generations.

Inorganic arsenic, the 20th most common element in the Earth's crust, is found in the environment in several chemical forms, and is well known for its ability to cause acute toxicity and death. It exists in several oxidation states, and those of the most interest to toxicologists are the trivalent and pentavalent forms, arsenite and arsenate. Humans are generally exposed to these compounds through contaminated water, food and air.

The chronic effects of arsenic on humans and animals include, but are not limited to: skin lesions, blackfoot disease, peripheral neuropathy, encephalopathy, hepatomegaly, cirrhosis, altered heme metabolism, bone marrow depression, papillary and cortical renal necrosis, diabetes and skin and lung cancer (335-337). While arsenic's toxicity has never been in question, controversy over its teratogenicity in humans continues unabated (13; 26; 338; 339). This is primarily due to the limited human data and questionably designed epidemiological studies (17-25). The primary flaw in most studies involving humans is that actual maternal exposure is not determined, rather environmental setting is substituted as a proxy. In the few studies in which maternal exposure was directly evaluated, measurements were not taken during the relevant embryological period of neural tube closure. These retrospective studies of a link

between arsenic exposure and increased risk of malformation measured maternal arsenic levels or environmental exposures long after the time when teratogens would be capable of inducing structural malformations. However, these studies proved valuable by demonstrating that arsenic readily crosses the human placenta and actually concentrates in the embryo/fetus and placenta during gestation (6; 14; 340). Acute prenatal arsenic exposure in humans can result in miscarriage and early neonatal death (27; 28).

In contrast to human studies, *in vivo* and *in vitro* experiments in laboratory animal species (mouse, rat, hamster and rabbit) have consistently shown arsenic to be a potent developmental toxicant and a teratogen, primarily inducing anterior neural tube defects (NTDs) (8-16). However, it should be noted that arsenic-induced teratogenicity was mainly demonstrated when the route of adminstration involved using injections (*intraperitoneal* and *intravenous*), and that arsenic doses used in these studies were high when compared to the doses likely to be experienced by humans in the environment (for detailed reviews the reader is referred to: (26; 339). In some studies, arsenic given orally or via inhalation revealed embryotoxic and/or teratogenic effects but at the doses presumably toxic for mothers (341-345). The relatively high doses of arsenic used in animal studies should not be a surprise, as lethal doses for laboratory animals are somewhat higher than the estimated lethal dose in humans. Based on clinical reports, the estimated minimal lethal dose for humans is about 1-3mg As/kg (346; 347), whereas available LD_{50} values for arsenite and arsenate in rats and mice range from 15 to 175 mg As/kg (336; 347). Thus, extrapolation from animal studies to humans is not trivial, and proper assessment of the developmental toxicity of arsenic will require much higher

quality human epidemiological studies and thoughtful investigation of potential modifiers to arsenic's teratogenicity in animal models. Taken together, these currently available data build a potential linkage between human arsenic exposure and elevated risks for neural tube and other congenital defects that bears further exploration (348). In this situation, it seems essential to fully understand the mechanisms by which arsenic may cause congenital defects, as this will enhance our ability to accurately predict the human health risks associated with arsenic exposure.

Folic acid enters cells via folate receptors (Folr's), also known as folate binding proteins, and the reduced folate carrier (RFC1), originally known as Slc19a1. Folr's are membrane bound and have both tissue- and cell-specific expression patterns (181). Murine Folr2, which is homologous to the human FOLR2, is variably expressed in most tissues (349; 350). To assess the role of Folr2 during embryogenesis, specifically during the period of neural tube closure, embryos lacking functional Folr2 alleles were generated by homologous recombination in embryonic stem cells (175). In this knockout strain, STOCK-Folr2tm1Fnn (Folr2), nullizygous embryos exhibited normal neural tube closure and had no grossly detectable abnormal phenotype. Plasma folate concentrations in these mice were unchanged relative to wildtype controls, and homocysteine levels were only slightly elevated, not reaching statistical significance (175). Repeated experiments consistently demonstrated that Folr2 nullizygous mice display increased sensitivity to arsenic-induced teratogenicity. Intraperitoneal injection of 9.6 mg As/kg as a sodium arsenate administered just prior to and during the onset of neural tube closure (embryonic (E) days 7.5 and 8.5) results in 40% exencephaly in nulls compared with

24% exencephaly in treated wild-types. This sensitivity to arsenic-induced NTDs was further enhanced by maintaining dams on a low-folate diet (351). When the Folr2 nullizygous dams were maintained on a low folate diet (0.3 mg/kg diet) and treated with arsenic as above mentioned, the level of exencephaly rose to 64%, while in the wildtype it remained relatively stable at approximately 25%.

Biotransformation of inorganic arsenic in most mammalian species requires a series of reduction and methylation reactions which facilitate arsenic excretion from the body (39). Recent studies revealed that intermediate trivalent arsenic species are extremely toxic, which suggest that methylation might not be the detoxification pathway (43; 352-354). In vitro study demonstrates that oxidation of trivalent arsenic species to pentavalent forms should be considered as a detoxification pathway (47).

In order to more thoroughly investigate the mechanism of arsenate-induced NTDs, we designed experiments in which highly sensitive Folr2 nullizygous mice were treated ip with a teratogenic dose of sodium arsenate just prior to the onset of neural tube formation process. This specific knockout mouse and arsenic exposure conditions were chosen as they previously produced a high incidence of exencephaly among the exposed embryos. We hypothesized that disruption of Folr2 in nullizygous mice disturbs methylation and excretion processes when challenged with high doses of arsenate, extending the time of embryo-exposure to arsenic. Results of our previous arsenic-induced teratogenicity study (351) and toxicokinetic study (unpublished data) on Folr2 knockout mice supports the above hypothesis. We have applied gene expression technology to study arsenic induced neural tube defects in these mice. This technique

allowed us to develop gene expression profiles within isolated regions of the target neural tissue that had been pre-selected for analysis. We investigated the gene expression changes induced by arsenic in the anterior part of neural tube in order to discover patterns that might shed light on the mechanism of arsenic's teratogenicity.

MATERIALS AND METHODS

Animals, Treatment and Collection of Samples

The STOCK-Folr2tm1Fnn ($Folr2^{-/-}$) null mice were housed in the Institute of Biosciences and Technology Vivarium. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet#8606, Ralston Purina, St. Louis MO) and were maintained on a 12-h light/dark cycle. Virgin females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning. The onset of gestation was set at 10 p.m. of the previous night, the midpoint of the dark cycle (355). Pregnant females were randomly assigned to receive sodium arsenate ($Na_2HAsO_4 \bullet 7H_2O$, Sigma-Aldrich Chemicals, St. Louis, MO) or water. On E 7:12 and 8:12, pregnant dams were injected *ip* with water solution of sodium arsenate at the dose of 9.6 mg As/ kg of body weight. Control dams were injected at the same time points with water for injection (USP, Abbott Laboratories Chicago IL) as a control treatment. The volume of each single injection amounted to 10 μL/g body weight. At three selected time-points: 8:15 (3 hours post-injection), 9:00 (12 hours post-injection) and 9:12 (24 hours post-injection), at least 4 control and arsenic treated dams were sacrificed and

embryos harvested. After a thorough gross morphological examination under a dissection stereomicroscope (Leica MZ95, Heerburg Switzerland) the embryos were staged according to somite number and advancement through the process of neural tube closure (Table 3.1). From these embryos, the anterior part of neural tube (cut off just in front of the otic pit) was dissected and transferred separately to a 600 μL plastic tubes containing 15 μL of RNAlater-ICE (Ambion). The samples were placed in -20°C for 24 hours to allow the tissue soak the RNA preserving compound, and then frozen at -80°C and kept until further processed.

Table 3.1. Embryos collected from Folr2$^{-/-}$ control and arsenic treated dams.

Gestational Day (day:hour)	Treatment	Litters (n)	Embryos (n)	Somites (mean ± SD)	Closure sites[#]				
					Open	I	II	III	IV
8:15	Control	5	28	7.9 ± 1.7	0	23	4	1	0
8:15	Arsenic	4	26	7.4 ± 1.6	0	26	0	0	0
9:00	Control	6	44	9.4 ± 2.8	1	33	2	8	0
9:00	Arsenic	6	39	8.5 ± 2.3	1	31	6	1	0
9:12	Control	4	30	20.4 ±1.8	0	0	0	0	30
9:12	Arsenic	4	28	17.7 ±3.8*	0	12	8	7	1

Sample Processing and Microarray Assay

No less than 6 embryonic neural tubes from separate dams (3 controls and 3 arsenic-treated) for each time-point were used for hybridization on commercially available CodeLink UniSet 10K Bioarray. Each array contains a broad range of genes (~10,500) derived from publicly available, well-annotated mRNA sequences. Every probe has been functionally validated against multiple tissues. A set of housekeeping genes has been included to be used for baseline normalization procedures. However, for a dataset of this size, a global scaling technique is definitely preferable. Array to array variation is said to be less than 8% across batches. Probe specificity is claimed by the manufacturer to be approximately 90% probe homology to target sequence, such that a 3bp mismatch would significantly reduce the hybridization intensity.

This translates into the ability to detect a 1.3 fold change in gene expression with 95% confidence or 2-fold with 98% confidence while differentiating between targets. Triplicate measurements through replicated array experiments further improve the confidence to 99.9%, which is crucial for settings with very large data sets such as microarrays. The sequence information of each probe (gene) is available on the http://www1.amershambiosciences.com/aptrix/upp01077.nsf/Content website. The 3-D gel provides support for 30-mers in a matrix that holds the probe away from the surface of the slide. This substantially reduces background and enhances sensitivity (1:300,000), allowing for the detection of one transcript per cell with 50-200 ng of poly A+ RNA.

Since the limited quantities of total RNA extracted from each anterior part of the embryo neural tube did not enable us to use standard procedures for probe labeling, we

utilized aRNA amplification methodologies as previously described by Eberwine and colleagues (356) with several modifications. Briefly, total RNA was extracted (PicoPure RNA isolation kit, Arcturus) and its quality was assessed on an Agilent 2100 Bioanalyzer using Pico LabChips (Agilent). To the RNA, concentrated to a final volume of 10μL in a Speedvac (ThermoSavant), 200ng of T7 RNA polymerase primer was added and the sample was denatured at 70°C and allowed to anneal slowly at 42°C. First and second strand cDNA synthesis was performed using 100 units of Superscript-II (Invitrogen) and DNA Pol I (Invitrogen). The DNA was isolated by a standard PCR purification kit (Qiagen) and concentrated using a Speedvac (ThermoSavant). The cDNA was then suspended in 8μL of RNAse-free water. The aRNA amplification synthesis was performed using Megascript (Ambion) at 42°C overnight in the presence of biotin-UTP. The aRNA was extracted using RNeasy kit (Qiagen) and the volume reduced to 10μL using a Speedvac. The net aRNA yield from each embryo neural tube was 10μg, and the aRNA quantity was determined using a fluorometer and the fluorescent nucleic acid stain RiboGreen (Molecular Probes). The aRNA was fragmented in 40mM Tris acetate, pH 7.9, 100mM KOAc and 31.5 mM MgOAc, at 94°C for 20 minutes. Hybridization, coupling of Alexa Fluor 647-streptavidin (Molecular Probes), and subsequent washes were performed according to manufacturer's protocol (Amersham Biosciences). The aRNA was mixed with buffer component A and B and then denatured at 90°C for 5 minutes. The sample was then injected into the hybridization chamber and sealed. Hybridization was allowed to go 18 hours at 37°C, while shaking at 300 rpm. Each slide was rinsed in TNT buffer (0.1M Tris-HCl pH 7.6,

0.15M NaCl, 0.05% Tween-20) at room temperature, followed by a wash at 42°C for 1 hour. Coupling of biotin labeled hybridized probe to dye labeled-streptavidin was performed in a 1:500 dilution of Alexa Fluor 647-streptavidin. Excess dye was removed by washing 4 times for 5 minutes each with TNT buffer at room temperature. Slides were rinsed in deionized water, spun-dry, and then scanned using an Agilent DNA Microarray Scanner.

Quantitative RT-PCR

TaqMan® Gene Expression Assays were used to validate microarray data for selected genes. Gene-specific probes and primer sets for Cox7c (Mm01340476_m1), Atp5k (Mm_00833200_g1), Hmox1 (Mm00516004_m1) and Sqstm1 (Mm00448091_m1) were purchased from Applied Biosystems (Foster City, CA). The RNA extracted from independent (different from those used for microarray assay), control and arsenic treated target tissue samples was reverse transcribed into cDNA with High Capacity cDNA Archive Kit (Applied Biosystems). 50ng cDNA of each sample was mixed with 10µl TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA) and 1µl probe and primer mixture in a total volume of 20µl in a 384-well plate. The assays were performed according to manufacturer's protocol on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). The thermo-cycling started with a 10 min denaturing at 95°C, followed by 40 cycles of 95°C for 15sec and 60°C for 1 min.

Statistical Analysis

Initial feature extraction from the images was performed using CodeLink Expression Analysis software v4.0 (Amersham Bioscience), and expression values for all 10500 genes were generated. Intensities for each individual gene were determined by the median intensity of all pixels within the spot's region. Subtraction of the median local background (computed from the subset of remaining pixels of the bounding box) yielded net intensities representing relative gene expression levels. In order to compensate for negative intensities, a FLOOR function with a value of 1 (range is 0-65535) was applied. For equidistant scaling, all values were logged with a base of 2 and the data were normalized using a global scaling method derived from single-channel normalization schemes used by Affymetrix analysis software. Expression intensities, ratios and gene ID's were then merged; the median of the expression values for each sample at the specified timepoint was used to generate the effective expression ratios representing the impact of arsenic on embryonic development in mice during the critical phase of neural closure. In other words, the comparison groups were the arsenic treated vs. the water injected Folr2 nullizygous embryos, with measurements taken over time. A thresholding function was employed to eliminate very weakly expressed genes. This, by definition, occurred where every intensity value contributing to the higher of the two gene expression levels was lower than the highest observed signal from negative control gene spots, plus four standard deviations derived from the distribution of the set of 481 negative control genes on the array. The remaining genes were sorted based on their differential expression and a boundary of 1.5-fold change in expression was used to

produce a list with considerably up- and down-regulated genes. In addition, a T-test has been applied, where a p-value of 0.05 ensured a consistent expression throughout the replicated arrays.

For hierarchical clustering, we used Hierarchical Clustering Explorer software v 3.0 (Human Computer Interaction Laboratory, University of Maryland, College Park), and the parameters were set for average linkage using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) and Pearson Correlation Coefficient.

The data were further analyzed based on gene ontology groups with the use of NCBIs GoMiner (Genomics and Bioinformatics Group, LMP,CRR National Cancer Institute) and ArrayTrack (Center for Toxicoinformatics, Division of Biometry and Risk Assessment, National Center of Toxicology Research, U.S. Food and Drug Administration), web-delivered Gene Ontology analysis tools to inquire about their relationships to known regulatory pathways. The gene ontology analysis produced clusters of differentially expressed genes according to biological processes, cellular component, and molecular function. Ontological enrichment analysis of all differentially expressed genes from all time-points collectively identified numerous ontological groups displaying significant enrichment ($p<0.005$).

Quantitative RT-PCR data were analyzed using SDS software v2.1 (Applied Biosystems, Foster City, CA). Mouse *Gapdh* gene was used as internal control. Standard curve method was used to generate quantitative values. Each reaction was replicated 3 times and the normalized mean value was used in final comparisons. The level of gene

expression was compared between arsenic treated and control neural tube samples. For that purpose the unpaired T-test was applied and the critical P value was set at 0.05.

RESULTS

Embryos Collected from Dams on E 8:15, 9:00 and 9:12

Majority of embryos collected on E8:15 was still in a "U" shape and had just started the process of neural tube closure (Theiler stage 13). There were no visible differences between the control and arsenic treated embryos. On E9:00 the embryos were undergoing axial rotation and most of them were already in a "C" shape (late phase of Theiler stage 13). The control embryos seemed to be a little bigger at this time-point and had on average one pair of somites more than the arsenic treated embryos. On E9:12 the progress in neurulation was more pronounced, with all the control embryos having closed anterior part of the neural tube (Theiler stage 14). In the arsenic treated group the embryos were developmentally delayed and most of them have closed the neural tube only at closure site I. At this time-point arsenic treated embryos showed significantly fewer somites than control embryos (Table 3.1).

Gene Expression Data Analyses

The entire gene expression data from this experiment (21 microarray chips representing genetic profile of neural tube samples from control and arsenic treated mouse embryos) were deposited on the NCBI Gene Expression Database. Genes that were differentially expressed across more than one time point after arsenic treatment in the anterior neural

tube are displayed. The data are presented as fold change after log2 transformation. Differential expression was defined as having at least a 1.5 fold change. Probe accession numbers and known gene symbols for the data presented include NM_010133 (En1), NM_009922 (Cnn1), AF209926 (Icmt), and NM_023850 (Chst1). Complete data can be accessed from this MIAME compliant depository database, omnibus web page http://www.ncbi.nlm.nih.gov/projects/geo/ via accession number GSE3412

In concert with the microarray data collection, we have performed an intensive bioinformatic analysis of the expression profiles in this unique genetic model of arsenic induced neural tube defect. In order to evaluate possible relationships between arsenic-induced malformations and gene-expression alterations, the anterior neural tube of mouse embryos was collected, processed, and the labeled aRNA was hybridized to microarray slides. A minimum of three biological replicates for each of the six tested groups were used: three time points (3, 12, and 24 hours after treatment) for controls and experimentals. A total of 21 CodeLink BioArrays were utilized for this experiment. Statistical parameters, as described above, were used to identify the most significantly altered up- or down- regulated genes for each time point. The threshold and T-test were applied (1.5 fold change, $P<0.05$), and those genes meeting this criterion at any time-point were graphed across the three time points. Out of 10500 gene elements that were present on the microarray slides, 233 were identified as differentially expressed in this study (Table 3.2.). Of these, 121 were up-regulated and 112 were down-regulated relative to controls with 31 being unknown. Analysis indicated that there were four genes (*Engrailed 1*, *Calponin 1*, *Isoprenylcysteine carboxylmethyltransferase*, and

Carbohydrate sulfotransferase 1) that displayed differential expression across multiple time points (Figure 3.1).

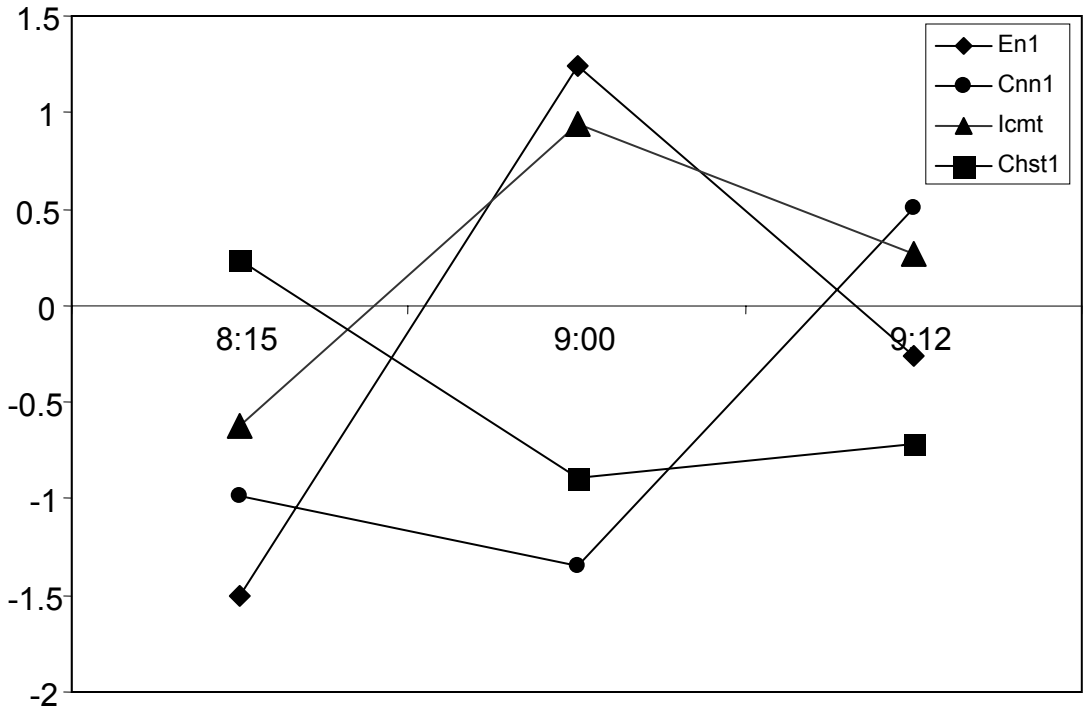

Figure 3.1. Genes with dynamic differential expression in the anterior neural tube from mouse embryos treated with arsenic

Table 3.2. Number of genes with changed expression level in the anterior part of neural tube from mouse embryos treated with arsenic.

Gestational Day (day:hour)	Up Regulated Genes	Down Regulated Genes	∑
8:15	18	37	55
9:00	87	60	147
9:12	16	15	31
∑	121	112	233

Hierarchical cluster analysis of differentially expressed genes was used to group samples, based on the degree of similarity of their gene expression profiles across all of the samples, separately for each time-point (8:15, 9:00 and 9:12). The results of this analysis showed that all control and arsenic samples (genetic profiles) were clearly assigned into two separated clusters (Fig. 3.3).

The data were further analyzed based on gene ontology groups with the use of GoMiner (Zeeberg et al., 2003). The gene ontology (GO) analysis produced clusters of statistically (p value <0.005) enriched differentially expressed genes according to their ontologies e.g. biological processes, cellular component, and molecular function. The ontology groups that were enriched (GO gene lists are available in the supplemental data) at each time point are shown in (Table 3.3).

Table 3.3. Gene ontology groups identified by GoMiner™ and ArrayTrack software in the neural tube samples from arsenic treated mouse embryos.

Gestational day (day:hour)	All Differentially Expressed Genes	Up-Regulated Genes	Down-Regulated Genes
8:15	protein kinase cascade*#	smooth muscle contraction*# nucleic acid binding* translation elongation factor*#	response to temp* eye morphogenesis*#
9:00	Ribosome*# inorganic phosphate transporter* caspase activator * apoptotic protease activator*	caspase activator activity*# apoptotic protease activator*# protein tyrosine phosphatase *# phosphoric ester hydrolase *#	ribosome* RNA binding* nucleic acid binding* mitochondrion* hydrogen ion transporter*# translation* threonine endopeptidase * proteasome core complex (euk)*# protein-membrane targeting*#
9:12	anion channel *	RNA splicing* anion channel*	
Globally changed	alpha DNA polymerase activity*, embryonic limb morphogenesis*, glycolysis*, hydrogen ion transporter activity*, regulation of I-kappaB kinase/NF-kappaB cascade*, response to hypoxia*, structural constituent of ribosome*, translation elongation factor activity*		

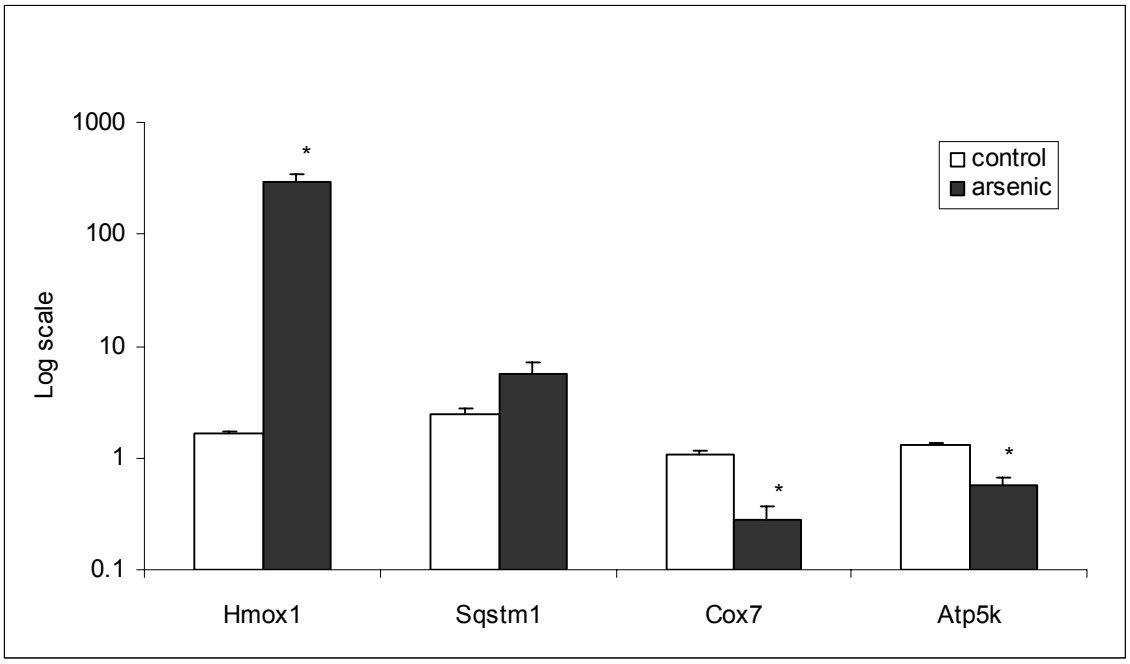

Figure. 3.2. Relative gene expression in the neural tube of the control and arsenic treated mouse embryos (qRT-PCR).
* significantly different comparing to control (t-test, $p<0.05$)

Quantitative Real-Time PCR (qRT-PCR). To independently validate gene expression results obtained by the microarray analysis we applied the TaqMan® Gene Expression Assay, which allows detection and quantification of mRNA. The expression levels of four genes that showed differential pattern of expression (two up- and two down-regulated genes) in neural tube samples from control and arsenic exposed embryos were quantified using TaqMan® RT PCR and compared to those levels determined by the microarray assay. More specifically we chose two genes (Hmx1 and Sqstm1) that were

upregulated by the arsenic exposure and two genes (Cox7 and Atp5k) down regulated (Figure. 3.2) The qRT-PCR results confirmed the microarray outcomes; Hmx1 and Sqstm1 were up regulated and Cox7 and Atp5k were significantly down regulated in the arsenic treated neural tube samples as compared to the control samples (Fig. 3.3). The overall, relative expression profiles (control/treated) of the four mRNAs revealed in qRT-PCR assay were found to be in a good agreement (correlation coefficient 0.97) with the expression profiles determined by microarray technique (Table 3.4).

Table 3.4. Comparison of relative ratios of gene expression obtained from qRT-PCR and microarray assays

Relative expression ratio control/arsenic treated	Hmox1	Sqstm1	Cox7	Atp5k
qRTPCR	0.01	0.43	3.96	2.32
Microarray	0.28	0.41	5.04	1.86

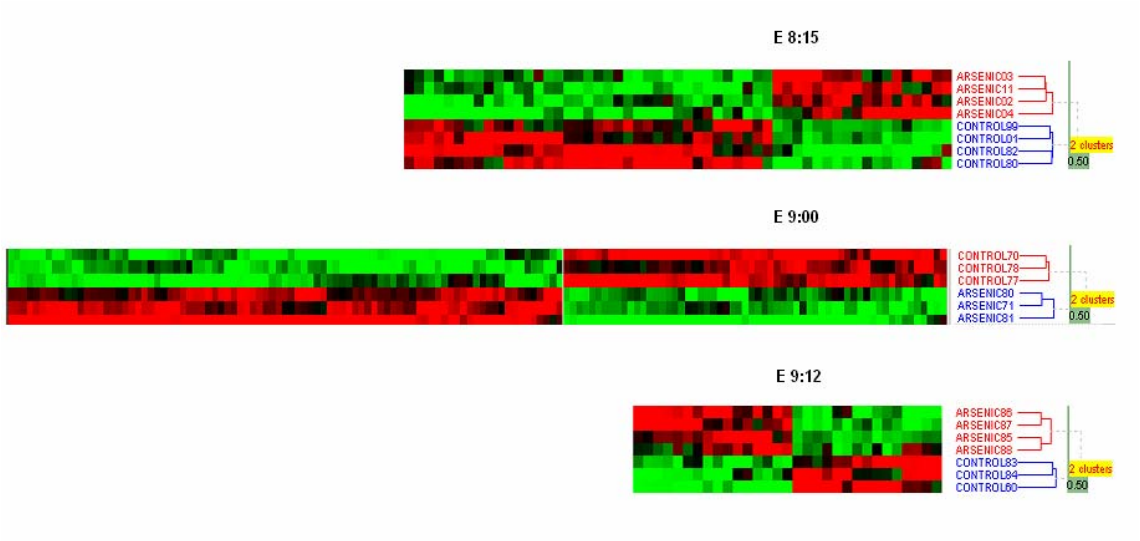

Figure 3.3. Hierarchical clustering of differentially expressed genes in the neural tubes of arsenic treated and control mouse embryos. The dendrogram is a group-based comparison (arsenic vs. control), the rows represent each array, while the columns represent a unique gene-specific oligonucleotide on the microarray. The colors indicate fluorescent signal intensity, red indicates an increase in expression levels after arsenic exposure, and green indicates a decrease. Note clean separation of the two treatment groups into two clusters, indicating the high level of reliability of the expression data.

DISCUSSION

Embryonic arsenic exposure via maternal ip injections leads to a well-described pattern of fetal malformations in mice (9; 16; 342; 351). It has also been demonstrated that arsenic interferes with normal gene expression in both cell and animal models, but the mechanism by which arsenic exposure induces malformation and alters gene expression remains to be elucidated. We suggest that these changes in gene expression are

responsible, at least in part, for arsenic induced malformations. This is supported by the fact that disrupting the expression of several arsenic responsive genes has previously been demonstrated to induce defects in mouse models. These genes included *engrailed 1 (En-1)*, *platelet derived growth factor receptor alpha (Pdgfrα)* and *ephrinA7 (EphA7)*. Inhibition of *En-1* by antisense targeting has resulted in craniofacial abnormalities (357), disruption of Pdgfrα was associated with craniofacial clefts, and cleft palate (358), and targeted gene disruption of *EphA7* has been shown to induce exencephaly (359). The gene expression analyses were designed in order to identify genetic alterations produced by teratogenic arsenic exposure. We observed that these changes were dynamic, in that the set of genes altered by arsenic depended directly on the time point following exposure. Gestational staging of embryos by somite numbers demonstrated that a delay in embryological development was induced by arsenic. Knowing that, in subsequent microarray studies, control and treated embryos were collected at the same time points and had developed to a comparable stage of development as determined by the number of somites. At the first two time points (E8:15 and 9:0) this developmental delay was very slight, as the vast majority of the control and arsenic treated embryos were in mid-neural tube closure at the closure site I. At that time, the average control embryo was slightly developmentally ahead of the arsenic treated embryos (0.5-0.9 somite pair) with respect to somitogenesis. Even though the difference between treated and control somite numbers were more pronounced at E9:12 (Table 3.1), we were still able to select control and treated embryos with comparable numbers of somites (18-19 pair of somites). Despite of the equivalent number of somites observed, the control embryos were

significantly more advanced in terms of the neurulation process, when compared to the As treated embryos. Analysis of the genetic changes that coincided with these developmental changes indicated essential housekeeping genes were altered by arsenic exposure (up regulated: Vdac1, Atp5b, Mphosph10, Snrpa and down regulated: Syncrip). Globally, alterations induced by arsenic included changes in genes providing housekeeping functions, morphogenesis, response to hypoxia, and regulation of I-kappaB kinase/NF-kappaB cascade (globally changed in Table 3.3).

It is generally hypothesized that multiple genes and pathways are responsible for complex birth defects (3). Therefore, the aim of the present study was to observe alterations in the gene expression of the anterior neural tube that precede the genesis of neural tube defects in arsenic-treated mouse fetuses. Characterization of gene alteration in the embryonic tissues that ultimately generates these malformations produced lists of up or down regulated genes, and these genes were then classified by gene ontologies. Genes identified by their relevance to toxicant response and dysmorphogenesis are described below, identified by the gene ontology group to which they belong, and clustered based on the timepoint they were shown to have altered expression.

3 Hours Post-Treatment

PKinase Cascade. Heme oxygenase 1 (Hmox1) is a hallmark for arsenic-induced stress (360). An essential enzyme in heme catabolism, it cleaves heme to form biliverdin a strong antioxidant. In addition to its substrate, heme, its activity is also strongly induced

by many agents causing oxidative stress. Microarray results indicated that Hmox1 was the most upregulated gene in the arsenic-treated anterior neural tubes.

Stress (Temp). Heat shock protein A1B (Hspa1b) is also known as 70kDa, Hsp70, hsp68, HSP70A1, Hsp70-1, and Hsp70.1. Heat Shock Proteins (HSPs) are a family of proteins that are induced by stressors such as heat and toxicants, and it is thought that the expression results in enhanced thermotolerance and chemotolerance. While the necessary induction of HSPs is well established during organogenesis in the rodent embryo, expression may also help provide protection against dysmophogenesis induced by toxicants. Work by (361) suggests that Hsp70-1 and Hsp70-3 are both necessary and sufficient to prevent arsenite-induced dysmorphology in early-somite staged mouse embryos. Both Hspa1b and Dnajc8 (another member of heat shock proteins, hsp40) were demonstrated to be highly upregulated at the earliest timepoint following arsenic treatment, and are known to participate together as a chaperone complex.

Morphogenesis. Aldehyde dehydrogenase family 1, subfamily A2 (Aldh1a2) is also known as Raldh1, Raldh2, Aldh1a7, RALDH-2 and AV116159. Retinaldehyde dehydrogenase converts retinal to retinoic acid (RA). RA expression is tissue and time specific, and is a developmental signal implicated in the formation of the neural axis. RA serves as a ligand for nuclear receptors, which influence transcriptional regulation of genes containing retinoic acid responsive element (RARE). At E8:12, RA can be detected in the mouse hindbrain. Raldh2-/- knockout mice die by E10:12 with heart defects (362), whereas excess of RA leads to defects in neural crest cell derived

structures such as craniofacies, heart, axial skeleton, and eyes. Aldh1a2 was upregulated in the arsenic treated samples, and may possibly drive additional RA synthesis.

Translation Elongation Factor. The sulfhydryl amino acid homocysteine is associated with an increased risk for cardiovascular disease and birth defects (363; 364). Homocysteine and methionine, both of which are part of the active methyl cycle, have also been shown to influence the expression of elongation factor-1delta (EF-1delta) in endothelial cells (365). This gene, a member of a multimeric complex regulating mRNA translation, was also differentially expressed after arsenic exposure. Furthermore, alterations in the s-adenosyl methionine to s-adenosyl homocysteine (SAM:SAH) ratio of the active methyl cycle have been demonstrated in Folr2 mice in a previous study (366). As in the endothelial model, these alterations in the active methyl cycle appear to induce the expression of EF-1delta and may thereby mediate accelerated synthesis of free sulfhydryl-containing proteins in response to oxidative stress (365).

12 Hours Post-Treatment

Ribosome. Ribosomal proteins are integrally involved in protein synthesis. The numerous proteins of the ribosomal subunits have multiple sequential steps that must be followed in order to properly initiate and translate mRNAs to proteins. The genes RPL10, RPL13, RPL14, and RPL18A are all found in the 60S ribosomal subunit. This subunit joins with the 48S initiator complex at the initiation complex of mRNAs, functionally forming the active 80S ribosome. RPL10, which is also known as QM, has been identified as a putative tumor suppressor, and it may also influence various signal

transduction pathways involving SH3 domain-containing membrane proteins (367). The gene RPL14 is also suggested to be a tumor suppressor gene (368). Therefore, the changes in expression of these ribosomal genes during development may directly alter ribosome biosynthesis required for cell proliferation and growth. These alterations may thereby increase the vulnerability of an embryo to a neural tube defect if there were delays in cellular proliferation and growth during neural tube closure. We observed such delays in growth in arsenic exposed embryos, and other studies have also demonstrated that inactivation of ribosome is similarly embryotoxic and teratogenic (369; 370).

Apoptosis. Arsenic trioxide (ATO) has been shown to induce differentiation and apoptosis in acute promyelocytic leukemia (APL) cells with concomitant upregulation of R1 and R2 APO2/TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) receptors (Akay et al., 2004; Liu et al., 2003). We observed alteration of Apo2/TRAIL in response to pentavalent arsenic exposure. These changes in Apo2/TRAIL may parallel what is seen in ATO exposed cells, in that ATO induces Apo2/TRAIL, activates caspase cleavage of BID, a pro-apoptotic BCL-2 family member, which depolarizes the mitochondrial membrane and releases Apoptosis-inducing Factor from mitochondria in a Bcl-2 independent fashion (371-373). Inducible expression of PYCARD/TMS1 has also been shown to inhibit cellular proliferation and induce DNA fragmentation (374). Since methylation of the CpG islands surrounding exon 1 of PYCARD/TMS1 has been indicated in controlling its gene expression (375), the biotransformation of arsenic may also directly influence the expression of PYCARD/MS1 by depleting the availability of methyl-groups and altering SAM:SAH (366).

24 Hours Post-Treatment

Anion transporter. Voltage-dependent anion channel (Vdac1) is also known as Vdac5. The voltage-dependent anion channel (VDAC) is a small, abundant pore-forming protein found in the outer mitochondrial membrane. It is hypothesized that mitochondrial membrane potential and the release of cytochrome c, initiating apoptosis, is regulated by the binding of BCL2 family of proteins to the VDACs (376). The BCL2 family of proteins' members may be anti-apoptotic or pro-apoptotic. Vdac1 is upregulated in the arsenic-treated samples.

ATP Synthase, H+ Transporting Mitochondrial F1 Complex, Beta Subunit (Atp5b). Atp synthase harnesses the energy of proton flux through the inner mitochondrial membrane to synthesize ATP from ADP during oxidative phosphorylation. Atp5b expression was upregulated in the arsenic-treated samples.

Global Analysis

A global analysis was performed on all differentially expressed genes at all time points. Global analysis using GoMiner indicated that the three most highly enriched GO groups were I-kappaB kinase/NF-kappaB cascade, oxidative phosphorylation, and eukaryotic translation elongation factor 1 complex.

The I-kappaB kinase/NF-kappaB cascade is activated by a wide range of cellular toxicants such as cytokines, reactive oxygen species (ROS), UV radiation, and many others. I-kappaB kinase, an inhibitor of NF-kappaB, is phosphorylated in the event of

cellular challenge. The phosphorylation marks the I-kappaB proteins for proteolysis via the ubiquitination pathway, allowing activation of the NF-kappaB complex. NF-kappaB, a nuclear receptor, translocates to the nucleus where it binds to NF-kappaB response elements in the regulatory regions of inducible genes, the expression of which help protect the cell from apoptosis. This gene cluster included Sqstm1, Birc2, Eef1d, Hmox1, Lgals1, and Tnfrsf10b.

Oxidative phosphorylation is the main method of energy production in the cell, using a high-energy electron passed through several respiratory enzyme complexes that pump H^+ out of the inner membrane of the mitochondria. This proton gradient is used by ATP synthase to form ATP from ADP and phosphate. ATP is essential to the completion of energetically unfavorable chemical reactions in the cell. The genes represented in this ontology group include the last three enzymes in the electron transport chain (Rfk, Bcs1l, Cox7c) and a two subunits of ATP synthase (Atp5k and Atp5b).

The eukaryotic translation elongation factor 1 complex ontology cluster consisted of Eef1a2 and Eef1d. These elongation factors control the recruitment of amino-acylated tRNA to the ribosome and regulate the translocation of the growing polypeptide (Hershey, 1991). Elongation factor 1a, one of the most abundant protein in the cell is also involved in cytoskeleton organization (377).

An evaluation of the relationship between the genes in the first two ontology groups suggests a clear pathway for cellular damage and response to this damage. Downregulation of the enzymes in the electron transport chain compromises oxidative phosphorylation, reducing available ATP, and perhaps more importantly, causes reactive

oxygen species (ROS) spilling, primarily consisting of superoxide anions into the cell. As these ROS overwhelm the cell's antioxidant capabilities, oxidative stress ensues. ROS disrupt cells through two main pathways: physical damage, and altered cellular signaling. In addition, damage from ROS may initiate a feedback loop, causing continued cellular damage. The genes from the I-kappaB kinase/NF-kappaB cascade were upregulated, and may indicate an attempt by the cell to ameliorate the damage induced by the ROS. The most highly upregulated gene observed in the i E8:15 neural tube array was the anti-oxidant Hmox1 (heme oxygenase), which is considered to be the hallmark of arsenic exposure. It has been proposed that damage to proteins induces heat shock proteins 40 and 70 (Hsp40 and Hsp70). When damaged proteins overwhelm the cell's ability to stabilize or degrade them, the sequestosome-ubiquitin sequestration system is induced, which isolates damaged proteins from the cytosol for later proteolysis. Microarray evidence suggests this scenario may be occurring in our model.

Arsenic can exert its toxic effect via several pathways: as a phosphate analog it interferes with phosphorylation reactions and competes with phosphate in ATP formation, through reactions with enzyme sulfhydryl groups it can inhibit many biochemical cell processes. There has been increasing evidence that arsenic toxicity is associated with free radical generation and that oxidative stress might be responsible for observed adverse effects. ROS not only can cause DNA and protein damage but also

cellular lipid peroxydation. Additionally they can serve as signaling molecules in the pathway leading to cell apoptosis (378). Results of our study seem to endorse that arsenic exerts its toxic effect via oxidative stress pathways. Analysis of gene expression in the neural tube of arsenic exposed embryos indicated that there was a significant dysregulation in a group of genes directly involved in the mitochondrial process of energy production. Disruption of this process leads to uncoupled oxidative phosphorylation, which ultimately causes oxidative stress. Analysis of microarrays also showed that arsenic strongly activated the I-kappaB kinase/NF-kappaB cascade which in turn launched several early response genes in response to oxidative stress. Additionally, besides confirming Hmox1 as a main marker gene of acute arsenic intoxication we revealed for the first time new target genes changed in expression by arsenic caused disruption of the oxidative phosphorylation process.

CHAPTER IV

REPRODUCTIVE CONSEQUENCES OF ORAL ARSENIC EXPOSURE DURING PREGNANCY

OVERVIEW

The second most common of all structural birth defects, neural tube defects (NTDs), affect approximately 2.6/1000 births worldwide, and 1/1000 births in the United States. Of the many environmental agents suspected of being teratogenic and capable of inducing NTDs, arsenic (As) commands intense scientific interest. We evaluated the teratogenicity of oral exposure on gestational day (E) E:7.5 and E:8.5 to As 4.8 mg/kg, As 9.6 mg/kg, and As 14.4 mg/kg (as sodium arsenate) in an inbred mouse strain, LM/Bc/Fnn, that does not exhibit spontaneous neural tube malformations. Control and As-treated dams (20 per treatment group) were weighed daily, and evaluated for signs of maternal toxicity. Fetuses were evaluated for soft tissue and skeletal malformations. There was no maternal toxicity as evidenced by differences in maternal body weight gain, liver, and kidney weights following As-exposure. The number of live fetuses affected with an NTD in each treatment group was: water treated control 0 (0.0%), As 4.8 mg/kg 1 (0.5%), As 9.6 mg/kg 7 (4.0%), and As 14.4 mg/kg 15 (8.2%), which exhibited a positive linear trend ($p < 0.0001$). There was also evidence for linear trends in the relationships between arsenic dose and congenital anomalies involving components of the axial skeletal (vertebral, $p < 0.0001$ and calvarial, $p < 0.0001$). In our model system, maternal oral treatment with As induced exencephaly and significantly increased

the frequency of axial skeletal variations and malformations in the offspring exposed in utero, without evidence of maternal toxicity.

INTRODUCTION

Birth defects are a significant health problem worldwide, affecting approximately 6% of births, and resulting in the death of at least 3.3 million children under the age of five each year (1). The second most common of all structural birth defects, neural tube defects (NTDs), affect approximately 2.6 out of every thousand births worldwide, and 1 per thousand live births in the United States (1; 2). NTDs represent a class of birth defects in which the embryo's neural tube fails to close prior to the end of the first month of pregnancy. Defects in the closure of the anterior neural tube result in exencephaly or anencephaly, in which the majority of the brain and surrounding tissues are absent. Failure of posterior neural tube closure results in spina bifida, a condition that often results in lower body paralysis and lack of bowel and bladder control.

While several distinct causes of NTDs have been identified, the etiology of the vast majority of NTDs remains frustratingly unexplained. There are numerous environmental exposures that are suspected of inducing NTDs in human embryos, yet definitive conclusions concerning the association between these potentially teratogenic environmental exposures and NTD risk remain difficult to reach. This is, at least in part, due to the relative rarity of both NTDs and the exposures of interest.

Of the many environmental agents suspected of being teratogenic and capable of inducing NTDs, arsenic (As) commands intense scientific interest. Naturally occurring

arsenic is an ubiquitous environmental toxicant known to contaminate water supplies around the world. In addition, anthropogenic sources result in tons of arsenic being released into the environment every year. Ten thousand metric tons of arsenic-based pesticides were applied in the U.S. every year from 1930 to 1980 (379). Rice grown in the southern United States has recently been observed to have higher levels of arsenic that Californian rice. This is suggested to be due to the practice of growing southern rice in fields previously used for cotton cultivation, as this crop was heavily treated with arsenic, and many of these fields remain heavily contaminated (380). In addition, monosodium methane arsenate is still one of the most common herbicides used on golf courses (381), and roxarsone, an arsenic-based feed additive, is heavily used in agribusiness to prevent parasites and enhance growth in swine and chickens (382). However, despite the pollution inherent in mining, industrial applications for gallium arsenide semiconductor chips, which are ubiquitous in household and automotive electronic products, continues to grow (383).

Studies in both humans and animals indicate that As crosses, and may accumulate in, the placenta at concentrations exceeding that of the maternal blood (6; 7). While As is a known laboratory animal teratogen, the few epidemiological studies of reproductive outcome following maternal As exposure are insufficient for properly assessing its teratogenic potential in humans. Available studies have lacked a sufficient cohort size to demonstrate associations with specific malformations, and the majority of human epidemiological studies have relied on proxy measures of exposure (e.g. distance from smelter) that are subject to varying degrees of misclassification. In the few studies

in which maternal exposure was directly evaluated, measurements were not taken during the relevant embryological period of neural tube closure (26).

Although the available data are insufficient to conclude definitively whether As is or is not a human teratogen, there is evidence suggesting that there may be a relationship between human in utero As exposure and adverse pregnancy outcomes. Acute high dose and chronic low dose As exposure during pregnancy has been associated with increased pre- and post-natal mortality (13; 27; 28). In addition, chronic low dose As exposure has been associated with low birth weight and developmental impairment (13). Several studies have also suggested an association between maternal exposure to As and an elevated rate of malformations in the offspring. At least two studies have provided evidence of an association between maternal As exposure in drinking water and risk of congenital heart malformations in exposed offspring (22; 29). In addition, indirect evidence for an association between As and NTD risk is provided by epidemiological studies linking NTD risk to maternal exposure to pesticides or proximity to agricultural areas (13; 31; 32). Given that As is environmentally persistent, and continues to be used in a range of agricultural products (e.g. pesticides, especially herbicides, growth enhancers, etc.) these studies provide a potential link between As and NTD risk.

Finally, evidence that maternal As exposure may be associated with an increased risk of congenital malformations, primarily NTDs, is provided by studies conducted using animal models. These studies have demonstrated that As crosses the placenta and preferentially accumulates in the neuroepithelium of developing hamster, mouse and

monkey embryos (33; 34), and that maternal As exposure is teratogenic. Furthermore, As is associated with an increased risk of NTDs when the exposure occurs specifically during the period of neural tube closure (26). However, criticism regarding the design of these studies has hampered the use of evidence from animal models for policy decisions. Common deficits ascribed to the early experimental animal literature on As teratogenicity include: use of environmentally non-relevant routes of exposure, lack of qualitative or quantitative assessment of maternal toxicity, lack of a dose-response design, insufficient number of animals per treatment group, incomplete identification of test substance, partial description of methods, and inadequate reporting of results (26). While many of these issues have been addressed as the field of teratology has matured, conflict remains over the issues of dose and route of As administration. It has been suggested that teratogenicity is only reached in laboratory animal studies when As doses are so high as to induce maternal toxicity, especially in the case of the few oral studies available for review (26). In the current study, we address concerns regarding dose and route of administration by evaluating the teratogenicity of oral exposure to As using an inbred mouse strain, LM/Bc/Fnn, that does not exhibit an underlying rate of spontaneous neural malformations, yet is highly sensitive to As-induced exencephaly.

MATERIALS AND METHODS

Test Agent and Treatment Regimen

Three treatment concentrations of arsenic: 4.8, 9.6, and 14.4 mg/kg of body weight of arsenic (in the form of sodium arsenate) for injection were prepared by dissolving sodium arsenate (Na2HAsO4 • 7H2O, Sigma-Aldrich Chemicals, St. Louis, MO) in Sterile Water for Injection (USP, Abbott Laboratories Chicago IL). Treatments were administered by oral gavage at a dose volume of 10 μL/g body weight. Dose levels were selected to bracket the 9.6 mg/ dose, which had been previously used in our laboratory for As teratogenicity studies that employed an intraperitoneal injection route of administration. The controls dams were gavaged with Sterile Water for Injection. Twenty pregnant females were assigned to each of the four experimental groups. On E7.5 and 8.5, pregnant dams were orally gavaged with either an arsenic solution or water.

Animals and Housing

The LM/Bc/Fnn mice were housed in the Institute of Biosciences and Technology Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet #8606, Ralston Purina, St. Louis MO). The mice were maintained on a 12-h light/dark cycle. Nulligravid females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning. The onset

of gestation was considered to be 10 p.m. of the previous night, the midpoint of the dark cycle (355).

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Observations and Measurements

All animals were observed once a day during the study period for morbidity and mortality. Maternal body weights were measured daily from E0.5 through E18.5, when the animals were euthanized. Weights of selected maternal organs were measured (gravid uterus, kidneys and liver). Litters were assessed by counting the number of implants, resorptions/dead, affected, and unaffected fetuses. A detailed external examination of each viable fetus was conducted. For each litter, half the fetuses were cleared with dilute potassium hydroxide solution, stained with Alizarin Red S, and examined for abnormalities in skeletal development. The remaining fetuses were fixed in Bouin's solution. Structures of the head were examined for malformations by Wilson sectioning. In addition, the thoracic and abdominal cavities were opened and the internal organs were examined grossly. Because cardiac malformations subsequent to in utero arsenic exposure have been observed in some mouse models in our laboratory, additional attention was paid to cardiac morphology. Specifically, the atria were dissected from the heart, and a transverse section was performed through the conotruncal region to identify possible malformations. This was followed by a careful opening of the right ventricle to determine if there were any ventral septal defects present. The type and incidence of external, visceral, and skeletal malformations were recorded.

Statistical Methods

One way analysis of variance (ANOVA) was used to evaluate differences, between treatment groups, in the mean values of continuously distributed variables (i.e. maternal body and organ weight, and weight gain). When the ANOVA provided evidence that the group means were not equal, differences in the mean values of each treatment group pair were evaluated. That is, the mean value for each treatment group was compared to that of the control group and the two other treatment groups. To account for the multiple pair-wise comparisons, the Bonferroni procedure was used to adjust the level of significance for each test. For these analyses, maternal organ weights (liver and kidney) were evaluated relative to maternal body weight.

The Kruskall-Wallace test was used to evaluate the differences, between treatment groups, in the distribution of the number of implantations and viable fetuses.

Differences in proportions, between groups, for categorical variables (i.e. resorption, NTD, skeletal malformation) were assessed using either the chi-square or Fisher's exact test. In addition, the Cochran-Armitage test was used to evaluate evidence for a linear trend (e.g. dose-response) in the relationship between arsenic dose and the occurrence of NTDs and skeletal abnormalities. When no abnormalities were observed in the control and the lowest As treatment group (i.e. vertebral abnormalities), these two groups were combined for the analysis of linear trend.

All statistical analyses were conducted using GraphPad InStat (version 3.06; GraphPad Software, San Diego, CA, USA), and the results of all tests were considered to be statistical significant when the p-value (or adjusted p-value) was less than 0.05.

RESULTS

Maternal Body Weight and Weight Change

On average, the initial body weights of dams in the arsenic treatment groups were not statistically significantly different from each other (Table 4.1). The initial body weights of dams in the groups receiving the two highest arsenic doses were also not statistically different from that of dams in the control group. However, the initial mean maternal body weight of dams receiving the lowest arsenic treatment was statistically significantly lower than that for control dams. Neither maternal weight gain throughout gestation (E0.5 – E18.5) (Figure 4.1) nor maternal weight gain from the onset of treatment (E7.5 – E18.5) were statistically different between any of the groups. Mean gravid uterine weights of dams receiving arsenic treatment all were significantly lower than control dams but were not statistically significantly different from each other. Maternal kidney and liver weights for treated dams were not significantly different from controls, nor were there significant differences between treatment groups with the exception of the liver weight, which was significantly lower in the lowest treatment group as compared to controls.

Table 4.1. Maternal body and organ weights of As-exposed groups relative to controls.

As Dose mg/kg	No. of Dams	Maternal Body Weight Gain (g)	Initial Maternal Body Weight (g)	Gravid Uterus (g)	Liver2 (g)	Kidneys2 (g)
Water	20	19.3 + 2.7	22.6 + 1.5	14.5 + 2.2	2.0 + 0.21	0.4 + 0.03
4.8	20	17.1 + 3.2	20.5 + 2.3*	10.4 + 2.3*	1.6 + 0.17*	0.35 + 0.04
9.6	20	17.7 + 3.4	21.1 + 1.7	11.1 + 2.6*	1.7 + 0.19	0.39 + 0.05
14.4	20	18.2 + 3.2	21.7 + 2.0	12.4 + 2.5*	1.8 + 0.24	0.39 + 0.04

1Data presented as mean + standard deviation.
2Data are present as raw averages, however, statistical analysis was performed as organ wt. relative to maternal body wt.
*Significantly different from control (Bonferroni, $p<0.05$)

Table 4.2. Developmental outcomes for As-exposed litters relative to controls.

As Dose mg/kg	No. of Litters	Implantations per litter (mean + SD)	No. of implants	No. of resorptions (%)	No. of Liveborn (%)	No. of Liveborn fetuses with NTDs (%)
Water	20	10.3 + 1.6	206	13 (6.3)	193 (93.7)	0 (0.0)
4.8	20	9.5 + 2.4	189	5 (2.6)	184 (97.4)	1 (0.5)
9.6	20	9.1 + 2.5	184	9 (4.9)	175 (95.1)	7 (4.0)*
14.4	20	9.9 + 1.6	197	13 (6.6)	184 (93.4)	15 (8.2)*

*Significantly different from control (Fisher's Exact, $p<0.05$)

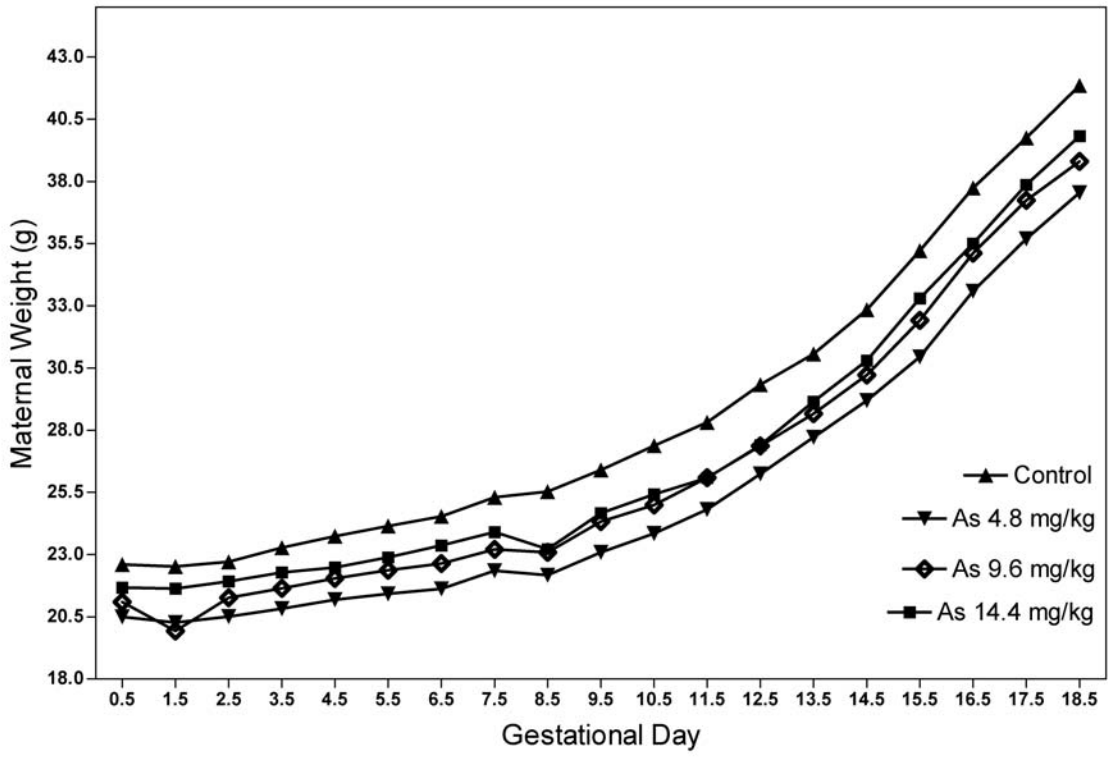

Figure 4.1. Maternal body weight gain throughout gestation.

Table 4.3. Skeletal variations and malformations observed in As-treated and control fetuses.

	As Dose mg/kg			
	Water	4.8	9.6	14.4
Fetuses (Litters) Examined	93 (20)	82 (20)	82 (20)	88 (20)
Sternebrae				
All sternebral abnormalities	7 (5)	20* (11)	23* (14)	26* (16)
Abnormal number (7 or 5)	2 (2)	4 (3)	11* (7)	8* (7)
Malformed (misaligned or bipartite)	5 (4)	18* (10)	13* (8)	20* (13)
Ribs				
All rib abnormalities	14 (8)	29* (14)	44* (20)	32* (14)
Abnormal number (11, 12 or 13)#	13 (7)	29* (14)	44* (19)	23* (11)
Malformed (fused, split, or bent)	2 (2)	0	2 (2)	10 (5)*
Vertebrae				
All vertebral abnormalities §	0	0	23* (15)	37* (16)
Atlas fused with occipital	0	0	5* (5)	4* (3)
Atlas fused with Axis	0	0	5* (3)	7* (5)
Atlas malformed	0	0	10* (9)	19* (13)
Axis malformed	0	0	11* (9)	14* (9)
Fusions between other cervical vertebrae	0	0	0	5* (3)
Vertebrae with irregular centra or arches	0	0	1 (1)	5* (4)
Calvariae				
All calvarial abnormalities §	0	33* (13)	53* (18)	68* (19)
Calvarial bones absent (exencephaly)	1	1 (1)	7* (5)	15* (9)
Occipital incomplete ossification	0	33* (13)	48* (17)	63* (18)
Interparietal incomplete ossification	0	10* (4)	34* (14)	46* (14)
Parietal incomplete ossification	0	8* (3)	6* (5)	16* (7)
Frontal incomplete ossification	0	3 (2)	0	5* (4)
Metacarpals				
Abnormal number (3)	0	6* (2)	4* (2)	2 (2)
Metatarsals				
Abnormal number (4 or 3)	10 (3)	22* (8)	14 (6)	21* (9)

*Significantly different from control (Fisher's Exact Test, $p<0.05$)
§Significant trend (Cochran-Armitage Chi-square Test for Trend, $p<0.05$)
Supernumerary 14th vertebrae are typically observed in this strain

Litters

The number of implantations and live fetuses (Table 4.2) was not statistically significantly different between groups. The rate of resorptions was also not significantly different between groups. The number of litters affected with an NTD in each treatment group was: Control (n=0), As 4.8 mg/kg (n=1), As 9.6 mg/kg (n=5), As 14.4 mg/kg (n=9). There was evidence of a significant linear trend in the number of affected litters with increasing dose of arsenic ($p < 0.0001$).

Malformations other than exencephaly and associated secondary facial disruptions were not observed during external or internal examination of Bouin's fixed fetuses (Fig 4.2). All exencephalic fetuses appeared to be of a comparable degree of severity, irrespective of As dose. Among fetuses with exencephaly, the eyes and ears were always present, and the supraoccipital, interparietal, parietal, and caudal aspects of the frontal bones were absent (Fig 4.3)

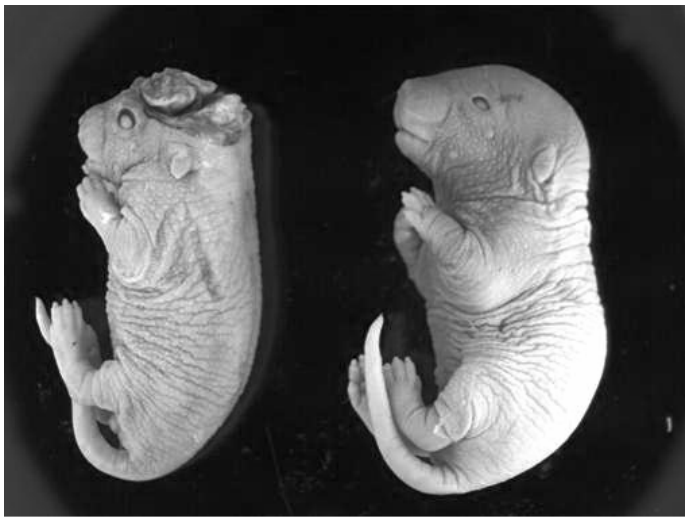

Figure 4.2. E18.5 fetuses: external morphology. On left, As treated fetus (14.4 mg/kg) displaying exencephaly with accompanying disruption of facial development (e.g.: protruding tongue, short muzzle). On right, control fetus with open eyes due to the lidgap M1 gene on the LM/Bc background

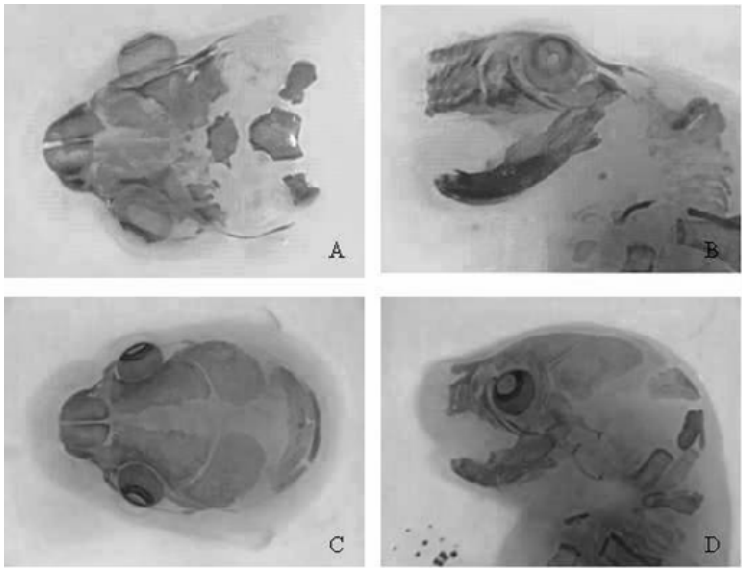

Figure 4.3. E18.5 fetuses: cranial skeleton. Top (A) and side (B) views of As treated (14.4 mg/kg) exencephalic fetus displaying loss of the supraoccipital, interparietal, parietal, and the caudal aspect of the frontal bones. Facial structures remain intact. Top (C) and side (D) views of control fetus displaying normal development.

Skeletal variations and malformations were observed at a very low rate in the control fetuses, with statistically significantly higher rates observed in the As-treated groups (Table 4.3). Similar numbers of skeletal malformations were found in As exposed fetuses with and without exencephaly. The observed abnormalities occurred primarily in the axial skeleton, although the tympanic bullae and hyoid bones were unaffected, and most frequently included delayed ossification of the cranial vault, fused and malformed vertebrae (almost exclusively C1 and C2 and T1 through T8), fused ribs (T1-T8), absence of normal supernumerary ribs, and supernumerary or absent sternebrae. It is important to note that the LM/Bc/Fnn mouse strain typically presents with a 14th pair of supernumerary ribs. There was evidence of a significant dose-response relationship between arsenic and abnormalities in components of the axial skeleton: vertebral ($p< 0.0001$), and calvarial ($p< 0.0001$).

DISCUSSION

In our model system, maternal oral treatment with As significantly increased the rate of exencephaly in the offspring exposed in utero, without evidence of maternal toxicity. The average litter size and resorption rates were similar in controls and all experimental groups. Further, there were no differences between controls and As exposed dams with regard to the average weight gained throughout gestation, or maternal organ weights (relative to maternal weights) with the exception of the liver weights of the dams in the lowest treatment group. While the cause of this difference is unexplained, it is difficult

to hypothesize a mechanism where the lowest treatment group would be exclusively affected.

Maternal arsenic treatment was associated with NTDs in offspring and there was evidence of a significant positive dose-response relationship. It is of interest that an exencephalic fetus was observed in the lowest treatment group. While the rate of NTDs in this groups was not significantly greater than in controls, a spontaneous neural tube defect has not been witnessed in the 30 years of close observation of this inbred strain in our laboratory (384). A significant positive dose-response was also seen for total As-induced vertebral and calvarial abnormalities.

As mentioned, it has been suggested that teratogenicity is only reached in laboratory animal studies when As doses are so high as to induce maternal toxicity, especially in the case of the few oral studies presented. However, as different strains of mice display markedly differing responses to arsenic's teratogenicity and toxicity, the assessment of arsenic in a limited number of strains is insufficient for the purposes of conclusively establishing or refuting the teratogenic potential of this agent. For example, the classic work performed by Hood in the Swiss-Webster mice of the CD-1 strain, demonstrated that a single I.P. dose of 9.6 mg/kg As (40 mg/kg of sodium arsenate) administered E8.5 results in 34% grossly malformed fetuses, 59% dead/resorbed, and the death of four out of ten dams, whereas oral treatment on the same day with 28.8 mg/kg of As (120 mg/kg of sodium arsenate) resulted in only 1% grossly malformed fetuses, 17% dead or resorbed, and the deaths of two out of nine dams (385). Studies performed in our laboratory also demonstrate differences in NTD rates due to route of

administration, but without evidence of maternal toxicity by either route. Specifically, treatment of LM/Bc/Fnn dams with the same I.P. route and dose as the classic Hood study (but treated on both E7.5 and 8.5) resulted in rates of exencephaly that approached 100%, whereas in the current oral treatment study markedly lower rates of exencephaly were observed. Hence, taken together these studies provide evidence of both strain and route dependent differences in teratogenicity and toxicity.

While an in-depth discussion of environmentally relevant dose selection in laboratory animal studies is outside the scope of this paper, it is important to note that the doses used in most laboratory animal studies are high when compared to doses likely to be encountered in exposed human populations (26; 339). Lethal doses calculated per kg of body weight for small laboratory animals are generally observed to be substantially higher than the observed and estimated lethal doses in humans: in summary, laboratory animals are generally less sensitive to toxicants. Arsenic is no exception to the rule. Based on clinical reports, the estimated minimal lethal dose of arsenic for humans is about 1-3mg As/kg (346; 347), whereas available LD50 values for arsenite and arsenate in rats and mice range from 15 to 175 mg As/kg (336; 347). While extrapolation from animal studies to humans is not trivial, laboratory animals are employed to mimic human biological responses. As such, it is important to consider the relevant toxic biological response in the selected laboratory animal and select for a dose that mimics the observed human response, rather than simply recreating the relevant environmental exposure. It may be appropriate to consider additional higher doses in studies of birth defects because

achieving an NTD rate of 2.6 per 1000 (which would mimic the current world rate of NTDs) would not provide an experimentally useful model.

It is generally hypothesized that multiple genes and pathways are responsible for complex birth defects (3). Arsenic's teratogenicity may be mediated through several pathways. As a phosphate analog, As may interfere with phosphorylation reactions and, through reactions with enzyme sulfhydryl groups, it can inhibit a multitude of biochemical cell processes. Arsenic has been observed to be highly mitochondriotoxic, an attribute that is exploited for use as a cancer therapeutic. Sequelae of mitochondrial disruption and oxidative stress are typically observed as a result of arsenic exposure: upregulation of heme oxygenase-1 (Hmox-1) and urinary 8-OHdG are considered markers of arsenic exposure. This is of interest because redox disruption and oxidative stress have long been thought to play an important role in the onset of some birth defects, although the exact relationship remains unclear and may involve a mix of signaling disregulation and macromolecular damage (87; 386). Our laboratory has previously performed a microarray study on anterior neural tube tissue collected from embryos exposed to arsenic at the onset of neurulation. While the results demonstrated

that arsenic induced mitochondrial disruption and oxidative stress, this study also revealed many genes whose altered pattern of expression has previously been shown to induce birth defects in mouse models. These genes included engrailed 1 (En-1), platelet derived growth factor receptor alpha (Pdgfra) and ephrinA7 (EphA7) (61).

Human exposure to arsenic during pregnancy appears poised to increase. Arsenic is currently an important part of industry in the United States, and its use worldwide is forecasted to increase as population-dense areas of the world such as India and China become ever more important players in the global industrial arena. It is an unfortunate circumstance that both of these countries already harbor pockets of significant natural arsenic contamination. Taken together, the ubiquitous nature of arsenic, the sensitivity of humans to arsenic toxicity and the teratogenicity of maternal oral arsenic exposure demonstrated in this, and other, studies builds a potential linkage between human arsenic exposure and elevated risks for neural tube and other congenital defects (348). This provides sound reason to perform more sophisticated human epidemiological studies to determine if environmental arsenic exposure poses a teratogenic threat to exposed human populations.

CHAPTER V

ARSENATE-INDUCED MATERNAL HYPERGLYCEMIA AND NEURAL TUBE DEFECTS IN A MOUSE MODEL

OVERVIEW

Epidemiological studies have linked environmental arsenic (As) exposure to increased risk of type 2 diabetes. A potential mechanism is revealed by studies both in vivo and in vitro suggest that arsenic is mitochondriotoxic, with acute exposure causing failure of GSIS, and subacute exposure causing profound changes in energy metabolism, resulting in type 2 diabetes. Periconceptional hyperglycemia is a significant known risk factor for having neural tube defect (NTD) affected pregnancies. The second most common of all structural birth defects, NTDs affect approximately 2.6/1000 births worldwide, and 1/1000 births in the United States. Suspected of being teratogenic and capable of inducing NTDs in animal models, As commands intense scientific interest. We evaluated the teratogenicity of maternal intraperitoneal exposure on As 9.6 mg/kg (as sodium arsenate) administered by intraperitoneal (I.P.) injection on gestational day (E) 7.5 and E:8.5 to in an inbred mouse strain, LM/Bc/Fnn, that does not exhibit spontaneous neural tube malformations. In addition, a range of compounds (LinBit insulin pellet, sodium selenate, NAC, L-Met, PBN) selected based on their potential to mitigate the effects of arsenic were evaluated to assed the effect on arsenate teratogenicity, maternal fasting plasma glucose, and insulin levels. Arsenate exposure cause NTDs (100%, $p<0.0001$) that were associated with maternal hyperglycemia (37% increase in FPG, $p<0.0048$) and

failure of GSIS, as demonstrated by maternal insulin levels that were not significantly different from water treated dams ($p>0.05$). All compounds tested significantly reduced the rate of NTDs ($p<0.001$ for all tests: sodium selenate reduced the NTD rate to 77%, PBN reduced the NTD rate to 74%, L-Met reduced the NTD rate to 68%, LinBit reduced the NTD rate to 45%, NAC reduced the NTD rate to 38%. With the one exception (PBN), none of the rescue treatments significantly altered the rate of resorptions in comparison to arsenate. NAC was successful in prevention of NTDs, though it did not restore maternal FPG or insulin levels to normal ($p>0.05$). The success of insulin in preventing NTDs provides evidence that arsenate-induced NTDs in this model are secondary to arsenate-induced hyperglycemia. Further, the protection against arsenate-induced NTDs provided by the antioxidants NAC, sodium selenate, and PBN suggest that embryonic oxidative stress is an important component of arsenate's teratogenicity. In summary, these experiments demonstrate the importance of arsenate's effects on mitochondrial disruption, failure of maternal GSIS, and to neural tube closure.

INTRODUCTION

Type 2 diabetes, characterized by hyperglycemia secondary to a relative lack of insulin, is a group of disorders whose multi-factorial etiology has both environmental and genetic components. While it has been known for nearly 30 years that environmental toxicant exposure may contribute to diabetes onset, the environmental toxicant arsenic has only recently come under scrutiny for its role in contributing to diabetes risk (92;

97). While arsenic is generally observed in six different chemical species having varying levels and differing mechanisms of toxicity, the forms primarily found in the environment are the inorganic trivalent and pentavalent forms, arsenite and arsenate, collectively referred to as Asi. Over the past few years, multiple epidemiological studies have demonstrated that total arsenic or Asi exposure from contaminated drinking water or in the workplace is a risk factor for type 2 diabetes (94; 95; 98; 106-108). While epidemiological studies have been limited in their characterization of the arsenic species responsible for this observed increase in diabetes risk, deliberate exposure to arsenate and arsenite alters glucose metabolism in humans, as well as in rodent and in vitro laboratory models. In fact, the most common side effect of arsenic trioxide (a trivalent compound) treatment for cancer is hyperglycemia (66). In rodent and β-cell models, both acute and sub-chronic exposure to both arsenite and arsenate has been shown to cause hyperglycemia or type 2 diabetes, respectively (103; 110; 111; 116).

Diabetes from environmental arsenic exposure is of interest because pre-gestational diabetes is associated with a two- to ten-fold increase in the risk of having an NTD affected pregnancy (132). NTDs represent a class of malformations wherein the embryo's neural tube fails to close, most commonly resulting in exencephaly or anencephaly, in which the majority of the brain and surrounding tissues are absent, or spina bifida, which often results in lower body paralysis and lack of bowel and bladder control (3). While epidemiological studies concerning arsenic's role in NTDs have proven inconclusive, embryonic arsenic exposure via maternal intraperitoneal (I.P.)

injection or oral exposure leads to a well-described pattern of fetal malformations in a variety of animal models, primarily characterized by NTDs (9; 16; 351; 387).

Maternal periconceptional hyperglycemia is teratogenic (128-130). Maternal blood glucose equilibrates with the embryo, dictating embryonic glucose levels until onset of fetal pancreatic function, well after neurulation is complete. As half of all pregnancies are unplanned, and women are typically past the critical time of neural tube closure when they initially detect a pregnancy, good prepregnancy glycemic control is essential in diabetic patients to reduce the risk of NTDs (131-133). Studies conducted using animal models provide further evidence that embryonic exposure to elevated glucose levels is mechanistically responsible for the increased risk of congenital malformation. Rodent models demonstrate that maternal hyperglycemia induced by direct injection of glucose or β-cell disruption from streptozotocin treatment results in exencephaly in the offspring, and explanted rodent embryos grown in high glucose media also exhibit a failure of neural tube closure (134). Recent work shows that gene inactivation of the glucose transporter, *Glut2*, in embryonic mice protects them from maternal hyperglycemia-induced malformations (388). A growing body of evidence collected over the past decade suggests that maternal hyperglycemia induces teratogenic oxidative stress in the exposed embryo, which is consistent with the well-established relationship between diabetes and oxidative stress (135).

Although the toxicity of arsenic varies with its chemical form and oxidation state, mitochondria are a consistent and primary target. Mitochondrial release of reactive oxygen species (ROS), especially H_2O_2, is thought to underlie much of arsenic's

toxicity. This hallmark of arsenic exposure provides a mechanistic link to disrupted glucose metabolism. Arsenite is more toxic than arsenate, and while they have some differing aspects to mechanisms underlying their toxicity, both have been demonstrated to be mitochondriotoxic, and able to disrupt glucose metabolism. Krebs first described arsenite's disruption of pyruvate metabolism in the 1930's (60), and arsenite and arsenate have since been demonstrated to reversibly inactivate nearly all of the Krebs cycle enzymes and to decrease expression of genes coding for portions of each of the electron transport chain components, resulting in mitochondrial release of H_2O_2 (61-64). Mitochondrial oxidative phosphorylation (OxPhos) links circulating glucose and insulin levels via ATP-dependent glucose stimulated insulin secretion (GSIS). Arsenite and arsenate can inhibit GSIS *in vitro* when administered to isolated pancreatic β-cell islets under glucose challenge (104). Additionally, excess H_2O_2 has been observed to reversibly repress many Krebs cycle enzymes, making pancreatic β–cells insensitive to GSIS, as demonstrated in *vitro*, as well as *in vivo* models (118-120). A byproduct of mitochondrial function, hydrogen peroxide is a negative feedback inhibitor of the Krebs cycle, preventing a toxic rise in oxidative radicals by controlling the rate of mitochondrial oxidative metabolism (118; 119; 124). It has a signaling utility that is especially potent in β-cells: they possess a weak antioxidant enzyme defense system, especially with regard to hydrogen peroxide-dismutating enzymes (125; 126).

We hypothesize that *in utero* arsenic exposure alters neurulation by disrupting maternal and embryonic mitochondrial function, inducing oxidative stress, which alters dependent biochemical pathways such as maternal ATP-dependent glucose sensing,

causing additional embryonic oxidative stress. In this study, we investigate arsenic-induced disruption of maternal glucose homeostasis and its role in the observed NTDs.

MATERIALS AND METHODS

Animals and Housing

The LM/Bc/Fnn mice were housed in the Institute of Biosciences and Technology Vivarium, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animals were maintained in clear polycarbonate microisolator cages and were allowed free access to food and water (Harlan Teklad Rodent Diet #8606, Ralston Purina, St. Louis MO). The mice were maintained on a 12-h light/dark cycle. Nulligravid females, 50–70 days of age, were mated overnight with males and examined for the presence of vaginal plugs the following morning, and the onset of gestation was considered to be 10 p.m. of the previous night, the midpoint of the dark cycle (355).

Test Agent and Treatment Regimen

Sodium Arsenate ($Na_2HAsO_4 \bullet 7H_2O$, Sigma-Aldrich Chemicals, St. Louis, MO) was dissolved in sterile water (Sterile Water for Injection, USP, Abbott Laboratories Chicago IL) to prepare a 9.6 mg/kg body weight dose of arsenic (in the form of sodium arsenate). This dose has been demonstrated to cause exencephaly in nearly all of the exposed offspring in this highly inbred mouse strain, without signs of embryotoxicity, which are increased resorption rates or decreased litter size. This enables us to make an

unequivocal assessment of the efficacy of the rescue compounds. As a commonly used dosing scheme in birth defects research, it additionally allows comparison of results between this, and other studies in this area. Both As and water control treatments were administered by intraperitoneal (I.P.) injection at a dose volume of 10 μL/g body weight. Treatments were administered on gestational day (E)7.5 and 8.5, immediately preceding, and at the onset of, neurulation, which generally occurs from E8.5-9.5.

Rescue Compounds

Five test compounds were selected to determine their ability to "rescue", or restore, the normal phenotype of the arsenic-exposed embryos. Pregnant arsenic-treated dams were randomly selected to receive one of the rescue compounds. Sodium selenate (Na_2SeO_4), L-methionine, and N-acetyl cysteine (Sigma-Aldrich) were administered daily from E0.5-10.5 by oral gavage as a standardized solution in sterile water such that they receive 10 μL of fluid per gram of body weight. The LinBit insulin pellet (LinShin Canada, Toronto, Canada) was implanted subcutaneously on E2.5 or 3.5, between onset of gestation and embryonic implantation. The pellet dissolves over a period of weeks, releasing insulin to the dam. N-tert-Butyl-α-phenylnitrone (Sigma-Aldrich) was injected I.P. on E7.5 and 8.5 as a standardized solution in sterile water such that the dams receive 10 μL of fluid per gram of body weight.

N-acetyl cysteine (NAC) (200 mg/kg) is an antioxidant that acts as a precursor of glutathione synthesis, a key substrate dismutating H_2O_2 to molecular oxygen and water. Sodium selenate (SS) (0.5 mg/kg) is thought to exert its effects through possible multiple

utilities: as part of selenocysteine, it is the active centre of antioxidant selenoenzymes such as glutathione peroxidase, has been demonstrated to have diverse effects on glucose homeostasis, and has been shown *in vivo* to methylate arsenic without the use of a methyltransferase. L-methionine (L-Met) (70 mg/kg) was selected to provide additional methyl groups for arsenic's biotransformation and help speed its excretion from the body. Insulin was selected to lower maternal circulating glucose levels, while N-tert-Butyl-α-phenylnitrone (PBN) (40 mg/kg) is a spin-trap, a compound that traps and stabilizes oxidative radicals, preventing deleterious molecular interactions. The two compounds most successful in rescuing the normal phenotype were characterized in further studies described below.

Fasting Plasma Glucose (FPG)

Fasting maternal plasma glucose was evaluated in tailsnip blood using a glucometer (Bayer Ascencia Elite XL) (Bayer HealthCare, LLC. Tarrytown, NY). Gravid dams were fasted for six hours prior to sample collection (beginning at 4:00 pm through 10:00 PM sample collection time) to allow for the evaluation of FPG levels at E:9.0, midpoint in neural tube closure. Experimental groups consisted of control, arsenic, arsenic/LinBit and arsenic/NAC treated dams.

Insulin ELISA

The amount of secreted insulin was determined to determine if observed elevations in maternal FPG were due to insulin resistance or failure of GSIS, and if the most

successful rescues were modifying the arsenic-induced effects. An enzyme-linked immunosorbent assay (ELISA) using antibodies that recognize mouse and rat insulin was used following the manufacturer's protocol (Rat/Mouse Insulin ELISA Kit, LINCO Research, St. Charles, Mo.) to evaluate tailblood samples (collected from gravid, fasting dams as described above for Fasting Plasma Glucose). Experimental groups consisted of control, arsenate, LinBit + arsenate, and NAC+ arsenate treated dams.

Observations and Measurements

Gravid dams were killed on E18.5, and litters were assessed by counting the number of implants, resorptions/dead, affected, and unaffected fetuses. A detailed gross external examination of each viable fetus was conducted.

Statistical Methods

Fisher's exact test was used to evaluate differences between treatment groups for litter outcomes (resorption/dead, NTD). The Kruskall-Wallace test was used to evaluate the differences, between treatment groups, in the distribution of the number of implantations. If $p<0.05$, a Dunn's Post-Test was performed. FPG and insulin levels were evaluating using 2-sided t-test ($p<0.05$).

All statistical analyses were conducted using GraphPad InStat (version 3.06; GraphPad Software, San Diego, CA, USA), and the results of all tests were considered to be statistical significant when the p-value (or adjusted p-value) was less than 0.05.

RESULTS

Comparison of Treatments

The highly inbred LM/Bc mouse strain is known to have a very low rate of spontaneous NTDs, and no NTD affected fetuses were observed in the absence of treatment with arsenate in these studies. Specifically, all test procedures (i.e. LinBit pellet placement, fasting, and additional maternal handling associated with blood collection for insulin testing) were evaluated in conjunction with a water control to determine whether they influence the outcomes of interest (see Table 5.1 for all results). The LinBit pellet, which is placed subcutaneously to provide exogenous insulin, was also evaluated, because historically it had been suggested that insulin treatment was responsible for the increased risk of malformations observed in the offspring of diabetic women.

Water treated dams implanted with LinBit displayed in increase in NTDs or resorption (Group A3) in comparison to water treatment alone (Group A2), ($p>0.05$ for both comparisons). Further, fasted water-treated dams tested for FPG (Group C9) displayed no increases in NTDs or resorption when compared to water-treated dams that were not fasted (Group A2), indicating that the six hour fast did not disrupt embryonic neural tube closure ($P>0.05$ for all comparisons). Similarly, water treated dams fasted for blood collection for insulin for testing (Group D14) displayed no increases in NTDs or

Table 5.1 Summary of litter and maternal outcomes following treatment.

Group		Administered Compounds	No. of Dams	Implants per Litter Mean ± SEM	No. of Resorptions (%)	No. of Fetuses with NTDs (%)	Maternal FPG (mg/dL) Mean ± SEM	Maternal Insulin level (mg/dL)
A) Controls	1	Arsenate	10	8.3±0.56	6(7.2)	77(100.0)	-	-
	2	Water	10	10.3±0.37	3(2.9)	0(0.0)	-	-
	3	LinBit	9	9.8±0.42	7(8.4)	0(0.0)	-	-
B) Rescue	4	NAC + As	11	9.7± 0.66	5(4.7)	39(38.2)	-	-
	5	LinBit + As	12	9.8±0.42	8(6.8)	51(46.4)	-	-
	6	L-Met +As	10	8.2±0.65	4 (4.9)	53(67.9)	-	-
	7	PBN +As	9	9.1±0.7	23(28.0)	43(74.1)	-	-
	8	SS +As	11	8.9 ±0.61	4(4.1)	72(76.6)	-	-
C) FPG	9	Water	9	9.2±0.32	3(4.8)	0(0.0)	85±6.3	-
	10	Arsenate	11	10.8±0.63	2(2.2)	91(100)	117±7.3	-
	11	LinBit	10	9.4±0.27	3(3.2)	0(0)	60±3.0	-
	12	NAC + As	13	8.3±0.58	9(8.3)	40(40.4)	106±3.8	-
	13	LinBit + As	11	7.7±0.79	16(18.8)	29(42.0)	82±9.8	-
D) Insulin	14	Water	8	8.5±0.68	0(0.0)	0(0)	98±5.1	0.445±0.043
	15	Arsenate	8	9.1±0.72	10(13.7)	63(100)	124±8.7	0.525±0.064
	16	NAC + As	9	7.9±0.79	7 (9.9)	32(45.1)	122±7.4	0.355±0.028
	17	LinBit +As	8	8.1±0.77	9(13.8)	28(43.1)	59.1±6.0	0.083±0.075

resorption when compared to water-treated dams that were not fasted (Group A2) ($p>0.05$ for all comparisons)

Arsenate Treatment

When treated with the arsenate regimen utilized in this study, nearly all of the exposed fetuses presented with NTDs. Arsenate-treated dams had significantly more NTD-affected fetuses (Group A1, 100%) than the water-treated controls (Group A2, 0%) ($p<0.0001$). However, the rates of resorptions were not significantly different in arsenate-treated dams when compared with water-treated dams ($p>0.05$).

Rescue of Arsenate-Induced NTDs

All compounds used to rescue the normal phenotype of embryos exposed to arsenate were successful compared to arsenate only, but the NAC and LinBit treatments were the most effective. Thus, NAC and LinBit treatment were selected for further investigation into maternal FPG and insulin levels.

As previously described, arsenate treatment (Group A1) exhibited a 100% rate of exencephaly. In the groups described above, SS+As (Group B8) reduced the NTD rate to 77% ($p<0.0001$); PBN+As (Group B7) reduced the NTD rate to 74% ($p<0.0001$); L-Met+As (Group B6) reduced the NTD rate to 68% ($p<0.0001$); LinBit+As (GroupB5) reduced the NTD rate to 45% ($p<0.0001$) and NAC+As (Group B4) reduced the NTD rate to 38% ($p<0.0001$). With the one exception, the rescue treatments did not significantly altered the rate of resorptions in comparison to arsenate. The single

exception, PBN, was associated with a significantly higher resorption rate of 28% ($p<0.0001$) as compared to arsenate (Group A1).

Maternal FPG and Associated Pregnancy Outcome

FPG testing was conducted in water (Group C9), arsenate (Group C10), NAC+As (Group C12) and LinBit+As (Group C13) treated dams that underwent a six hour fast. In all groups, tailblood was tested at the midpoint of neural tube closure. With one exception, fasted dams displayed no differences for NTD or resorption rates when compared to the similarly-treated dams that were not fasted (i.e. arsenate alone vs. fasting, (A1 vs. C10); water alone vs. fasting, (A2 vs. C9); LinBit alone vs. fasting, (A3 vs. C11); and NAC+As vs. fasting, (B4 vs. C12)). LinBit+As+fasted dams had a significantly higher rate of resorptions (Group C13, 19%) when compared to the LinBit+As dams that were not fasted (Group B5, 7%) ($p<0.0001$). It is notable that fasting did not diminish the effects of the NAC or LinBit on the rates of arsenate-induced NTDs (Table 5.1, Group C).

The FPG levels of dams treated with arsenate (C10, 117 mg/dL) were significantly higher than dams treated with water (Group C9, 85 mg/dL) (Two-Tailed T-test, $P=0.0048$). Dams receiving LinBit in addition to arsenate (Group C13, 82 mg/dL) had FPG levels similar to dams receiving water controls (Group C9, FPG 85 mg/dL) ($p>0.05$). In contrast, in NAC treated dams (Group C12) FPG levels (106 mg/dL) remained high, and were significantly different from water treated dams ($p<0.0065$). LinBit+water (Group C11) was evaluated to confirm that LinBit treatment alone was

sufficient to lower FPG in water-treated dams. LinBit+water treatment (Group C11) successfully lowered the FPG (60 mg/dL) below the FPG associated with water treatment ($p<0.0019$). As indicated earlier, the low FPG associated with this treatment was not associated with an increase in NTD or resorption rates in comparison to water alone.

Maternal Insulin

Insulin testing was conducted in water+FPG+insulin (Group D14), arsenate+FPG+insulin (Group D15), NAC+As+FPG+insulin (Group D16) and LinBit+As+FPG+insulin (Group D17). Dams underwent a six hour fast in order to evaluate maternal blood glucose levels midpoint in neural tube closure, as well as additional handling to collect blood for an ELISA. Dams in this groups displayed no differences in NTD or resorption rates when compared with the similarly treated dams that were not fasted or tested for insulin levels, indicating that the additional handling and blood collection was neither teratogenic nor embryotoxic ($P>0.05$ for all comparisons).

All FPG levels associated with this insulin testing were similar to FPG levels in dams not tested for insulin, though they were slightly higher overall, possibly reflecting the stress of additional handling. Most importantly, arsenate treatment (Group D15) continued to be associated with a significantly higher FPG (123 mg/dL) in comparison to water treatment (Group D14) (FPG 98 mg/dL). However, there was no difference between the insulin levels in these same groups, indicating that elevated levels of

circulating glucose caused by arsenate failed to stimulate compensatory insulin secretion ($p>0.05$). Similarly, the FPG (122 mg/dL) associated with the NAC treatment (Group D16) was significantly higher than the water treatment (Group D14), with no difference in insulin levels. The LinBit treatment alone (Group D17) significantly lowered the FPG (60 mg/dL), and the exogenous insulin, as expected, elevated the insulin levels in comparison to water treatment (Group14).

DISCUSSION

This study provides evidence that some arsenate-induced NTDs in this model are secondary to arsenate-induced hyperglycemia. Further, these experiments highlight the importance of arsenate-induced failure of mitochondrial activation and GSIS, and subsequent oxidative stress, to neural tube closure. While all selected rescue compounds significantly rescued the normal embryonic phenotype, NAC and insulin were the most effective. In particular, the success of maternal administration of insulin in rescuing arsenate-induced NTDs suggests that an important component of the teratogenicity of arsenate is mediated through maternal hyperglycemia in this model. The resulting teratogenic effects of maternal hyperglycemia-induced oxidative stress is highlighted by the success of maternal administration of NAC, an antioxidant and precursor of glutathione synthesis, to rescue the normal embryonic phenotype, without restoring the normal maternal GSIS and FPG. Further, the oxidative stress component of arsenate-induced NTDs is shown by the rescue achieved by the administration of PBN, a radical trapping agent which inactivates toxic radical species was highly effective as an inhibitor

of arsenate-induced NTDs. However, it also significantly increased the number of resorptions and therefore was not further investigated.

Due to the diverse biochemical effects of arsenate, no single pathway or experimental model comprehensively describes its toxicity. We suggest that arsenate has both maternal and embryonic effects that ultimately prove teratogenic in our model. Maternally administered arsenic concentrates in the embryonic neuroepithelium of both rodents and primates (33; 34). While arsenate causes profound mitochondrial disruption affecting both the dam and embryo, this has important implications for maternal blood glucose regulation, whose circulating glucose equilibrates with the embryo's during early developmental timepoint. The embryo is thus exposed to two separate, but related, oxidative insults: direct mitochondrial damage via in utero arsenic exposure, and oxidative stress due to the hyperglycemic milieu.

Many types of mitochondrial disruption have been associated with diabetes. While a limited number of types of monogenic mitochondrial diabetes have been discovered (loss of function of mitochondrial TRNAs, nuclear encoded mitochondrial transcription factor A, TFAM, TCA cycle enzyme α-ketoglutarate dehydrogenase), the much more common type 2 diabetes is thought to be due to complex gene-environment interactions. Mitochondrial disruption involving such disparate factors as mitochondrial number or mtDNA copy numbers and gain or loss of function of uncoupling protein 2 (UCP2) have been associated with type 2 diabetes (389).

Mitochondria are a common target of both environmental toxicants as well as many pharmaceuticals. Many pharmaceuticals, household, and industrial chemicals can impair

insulin secretion. While these exposures are not generally thought to cause frank diabetes, they may initiate diabetes onset in individuals who are predisposed, such as those with insulin resistance. A sampling of compounds and pharmaceuticals recognized to induce diabetes (and their application) follows: Vacor (rat poison), pentamidine (antimicrobial), nicotinic acid (vitamin b_3), glucocorticoids (steroid), thyroid hormone, diazoxide (vasodilator), β-adrenergic agonists (calcium channel modifiers), thiazides (diuretic), dilantin (antiepileptic), α-interferon (cancer and viral therapeutic). Interestingly, thiazides are associated with NTDs, and dilantin is associated with cleft palate, another craniofacial malformation associated with diabetic pregnancy (89). Valproate, a known risk factor for NTDs, is an antiepileptic and atypical antipsychotic, which are widely observed cause hyperglycemia (90; 91).

The first environmental contaminant to be associated with an increased diabetes risk was found in the 1970s, when carbon disulfide exposure's relationship to diabetes was reported (89; 92). Interest in other environmental contaminants and their potential to increase diabetes risk is relatively recent, and has focused on arsenic, dioxin, and nitrates

(93-96). For a comprehensive review of this subject, see work by Longnecker and Daniels (97). At the time publication of Longnecker's work, while no exposures were conclusively linked with increased diabetes risk, several occupations and occupational exposures were identified that may have contributed to diabetes onset. Evaluation of studies was hampered by aspects of study design that limit the power of the investigation including: use of glucosuria or diabetes death as diagnostic criteria, lack of adjustment for possible confounders in some studies, and failure to consider both type 1 and type 2 diabetes as possible adverse health outcomes. Data concerning arsenic and TCDD were most suggestive of an association with diabetes, while nitrates, nitrites, and N-nitroso compounds had a weaker, but not null, association. Further epidemiological and laboratory research concerning arsenic's association with increased diabetes risk has since been published (98-105). In summary, as we continue to dissect the multifactorial causes of these complex diseases, diabetes and NTDs, environmental exposures to mitochondriotoxic compounds warrant further investigation.

CHAPTER VI

SUMMARY AND CONCLUSIONS

SUMMARY

Gene expression technology was used to investigate arsenate-induced NTDs in Folr2 nullizygous mice, a mouse strain with an engineered deletion of a gene involved in the receptor mediated folate pathway. These mice were injected intraperitoneally with sodium arsenate at the beginning of the neural tube formation process, resulting in a high incidence of NTDs in the exposed embryos. Gene expression was evaluated in embryonic anterior neural tube. This allowed us to study arsenic-induced changes in patterns of gene expression that may contribute to the development of neural tube defects in these mice. Using extensive data analysis approaches including hierarchical clustering and gene ontology analysis, we identified several candidate genes, as well as important ontology groups that may be responsible for arsenic's teratogenicity. Changes in the expression of several genes in response to arsenic treatment in our model had been previously demonstrated by others to also induce NTDs in murine model systems. These include: *engrailed 1 (En-1)*, *platelet derived growth factor receptor alpha (Pdgfrα)* and *ephrinA7 (EphA7)*. Several gene ontology groups that could be implicated in arsenic's underlying teratogenicity were also revealed. They include: morphogenesis, oxidative phosporylation, redox response, and regulation of I-kappaB kinase/NF-kappaB cascade. Additionally, we revealed new target genes which may be responsible for arsenic disrupted oxidative phosphorylation.

Oral arsenate exposure was investigated to determine if this common environmental route of exposure was teratogenic. This was investigated in an inbred mouse strain, LM/Bc/Fnn, that is known to have a very low rate of spontaneous NTDs. There was no maternal toxicity as evidenced by differences in maternal body weight gain, as well as liver and kidney weights following arsenate exposure. Oral maternal arsenate exposure resulted in NTDs in the offspring, and dose-response exhibited a positive linear trend ($p< 0.0001$). The dose, number of live fetuses affected with an NTD, and percent of litters affected were: water treated control, 0, (0.0%): As 4.8 mg/kg, 1, (0.5%): As 9.6 mg/kg, 7, (4.0%): and As 14.4 mg/kg, 15, (8.2%). There was also evidence for linear trends in the relationships between arsenic dose and congenital anomalies involving components of the axial skeletal (vertebral, $p< 0.0001$ and calvarial, $p< 0.0001$).

Environmental arsenic exposure has been associated with increased diabetes risk in epidemiological studies. Because hyperglycemia, the hallmark of diabetes, is teratogenic in humans as well as in rodent models, we investigated whether the arsenate-induced NTDs involved disruption of glucose metabolism. Arsenate exposure cause NTDs (100%, $p<0.0001$) that were associated with maternal hyperglycemia (37% increase in FPG, $p<0.0048$) and failure of GSIS, as demonstrated by maternal insulin levels that were not significantly different from water treated dams ($p>0.05$). All compounds tested significantly reduced the rate of arsenate-induced NTDs ($p<0.001$ for all tests: sodium selenate reduced the NTD rate to 77%, PBN reduced the NTD rate to 74%, L-Met reduced the NTD rate to 68%, LinBit reduced the NTD rate to 45%, NAC reduced the

NTD rate to 38%. With the one exception (PBN), none of the rescue treatments significantly altered the rate of resorptions in comparison to arsenate. NAC was successful in prevention of NTDs, although it did not restore maternal FPG or insulin levels to normal ($p>0.05$).

CONCLUSIONS

Analysis of gene expression in the neural tube of arsenic exposed embryos indicated that there was a significant dysregulation in a group of genes directly involved in the mitochondrial process of energy production. Disruption of this process leads to uncoupled oxidative phosphorylation, which ultimately causes oxidative stress. Analysis of DNA microarrays also showed that arsenic strongly activated the I-kappaB kinase/NF-kappaB cascade, which in turn, launched several early response genes in response to oxidative stress, such as Hmox1. In addition, we revealed for the first time new target genes that was altered in their expression by arsenic induced disruption of the oxidative phosphorylation process. The oral route of exposure was determined to be teratogenic in our LM/Bc/Fnn mouse strain, with a positive dose-response. Maternal oral treatment with arsenate induced exencephaly and significantly increased the

frequency of axial skeletal variations and malformations in the exposed offspring. The fetal malformations were produced in the absence of maternal toxicity. The I.P. route of exposure used in the LM/Bc/Fnn mice caused NTDs that were associated with maternal hyperglycemia. The success of insulin in preventing these arsenate-induced NTDs provides evidence that arsenate-induced NTDs in this model are secondary to arsenate-induced hyperglycemia. Further, the protection against arsenate-induced NTDs provided by the antioxidants NAC, sodium selenate, and PBN suggest that embryonic oxidative stress is an important component of arsenate's teratogenicity. These experiments demonstrate the importance of arsenate's effects on mitochondrial disruption, failure of maternal GSIS, and to neural tube closure.

The results of these studies are consistent with established mechanisms of arsenate's toxicity. Results of our study strongly suggest that arsenic exerts its toxic effect via oxidative stress pathways.

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